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(54) **PROTEINE ASSOCIEE AU TRAF4**

(54) **TRAF4-ASSOCIATING PROTEIN**

(57) The present invention relates to a factor associated with TNF receptor associated factor 4 (TRAF4), novel proteins TRAF Four Assiociated Factor 1 and 2 (TFAF1 and TFAF2) and novel genes encoding TFAF1 and TFAF2. TFAFs of the present invention are considered useful for cancer control.

GENE

9 x

TRAF4-ASSOCIATING PROTEIN

Field of the Invention

The present invention relates to a gene encoding a novel protein capable of associating with TNF receptor associate factor 4 and said protein itself. It is highly possible that this protein is closely related to the malignant transformation system, and the protein is expected to control tumors.

Background of the Invention

Tumor necrosis factor (TNF) is a cytokine which has been found to induce hemorrhagic necrosis of tumors. It has also been found that this factor activates cells and stimulates cell proliferation. Furthermore, it seems profoundly involved in inflammatory symptoms.

TNF receptor (TNFR) is the receptor present on the cell surface which binds TNF. It is known to exist in two types, TNFR1 (Mw 55 kDa) and TNFR2 (Mw about 75 kDa). TNF signaling is divided in two pathways. One induces apoptosis mediated by TNFR1; the other stimulates the cell proliferation by activating the nuclear factor kappa B (NF-κB) mediated by TNFR2. In the latter, a factor involved in transducing the signal from TNFR to the nucleus is the TNF receptor associated factor (TRAF). The molecules binding to the intracellular domain of TNFR2, TRAF1 and TRAF2, were first discovered in 1994 (Roche, M. et al., Cell 1994, Aug. 26; 78 (4): 681-692). For TRAF, a group of structually homologous genes, a family of six types, TRAF1 through TRAF6, have now b en isolated. Of these, TRAF1, TRAF2, and TRAF3 (Sato, T. et al., FEBS Lett, 1995, Jan 23; 358 (2): 113-118), TRAF5

(Ishida, T. K. et al., Proc. Natl. Acad. Sci. USA 1996, Sep.3; 93 (18): 9437-9442), and TRAF6 (Ishida, T. et al., J. Biol. Chem. 1996, Nov. 15; 271 (46): 28745-28748) have a variety of target genes such as genes of cytokines and cell adhesion molecules, and are involved, directly or indirectly, in activating of NF-κB, one of the objectives for an anti-inflammatory agent [Nakano, H. et al., J. Biol. Chem., 1996, Jun. 21; 271 (25): 14661-14664, Cao, Z. et al., Nature 1996 Oct. 3; 383 (6599): 443-446, Song, H. Y. et al., Proc. Natl. Acad. Sci. USA, 1997 Sep. 2; 94 (18): 9792-9796, Roche, M. et al., Science 1995 Sep. 8; 269 (5229): 1424-1427].

TRAF4 (a C-rich motif associated with RING and TRAF; once called CART1), a member of the TRAF gene family, is a molecule cloned by applying the subtraction method for normal cells and breast cancer cells [Tomasetto, C. et al., Genomics 1995, Aug. 10; 28 (3): 367-376]. In fact, it has been reported that the TRAF4 gene is amplified and overexpressed in a portion of primary culture of cells derived from breast cancer patients [Bieche, I. et al., Cancer Res. 1996 Sep. 1; 56 (17): 3886-3890]. In this respect, TRAF4 is unique compared with other TRAFs which have been isolated as molecules associating with the TNFR superfamily. Furthermore, TRAF4 differs from other TRAFs in that it has a nuclear location signal as a motif in its amino acid sequence and has been reported to be actually localized in the nucleus (J. Biol. Chem., 1995 Oct. 27; 270 (43): 25715-25721). The cDNA structure of TRAF4 is shown in Fig. 9. TRAF4 also differs functionally from other TRAFs in that its involvement in the NF-κB activation has not been reported. Recently, the transcription of

TRAF4 has been reported in the central and peripheral nervous systems of mouse embryos. It has also been confirmed in the hippocampus and olfactory bulb to maintain the differentiation capability in adults [Masson, R. et al., Mech. Dev., 1998 Feb. 1; 71 (1-2): 187-191]. Even from the previous limited reports, TRAF4 differs from other TRAFs in the method of isolation, intracellular localization and function, and is noteworthy protein, in spite of having amino acid sequence homologous to those of other TRAFs. However, little has been known of TRAF4, so it is awaiting further clarification.

For example, no one has reported factors associated with TRAF4 and few have report on its function. From the characteristics above, TRAF4 is presumably localized in the cellular nucleus, near the downstream of the signal transduction pathway of cell proliferation and is presumably one of the oncogenes which causes breast cancer by its amplification and overexpression for some reason.

If any factor inhibits or enhances the function of TRAF4, such factor is probably a factor associated with TRAF4. Identifying factors associated with TRAF4 is a very important objective for explaining TRAF4 functions in detail.

Summary of the Invention

One objective of the present invention is to isolate an unknown factor, a TRAF4-associated factor, and clarify its structure and functions. Another objective of this invention is to provide a novel treatment and diagnostic technique for cancer based on this unknown associated factor.

The present inventors searched for a TRAF4-associated protein

using a two-hybrid system of yeast. They succeeded in isolating a novel gene from a human placenta cDNA library as DNA encoding a protein capable of associating with the TRAF domain located at the carboxyl-terminal region of TRAF4. In other words, this invention provides a novel DNA sequence and a novel protein encoded by said sequence as described below. The present inventors designated the expression product of this novel gene as TFAF, TNF receptor associated factor 4 associated factor (TRAF Four Associated Factor). This novel protein, TFAF, discovered by the present inventors, includes two family members, TFAF1 and TFAF2. In addition, this invention provides a method for obtaining these DNAs and proteins and their use. Specifically, the present invention relates to:

(1) a protein comprising the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4, or a protein having one or multiple amino acids substituted, deleted or added in the amino acid sequence of said protein, and capable of associating with TNF receptor-associated factor 4;

(2) a protein encoded by a DNA hybridizing with DNAs comprising nucleotide sequences of SEQ ID NO:1 or SEQ ID NO:3, and capable of associating with TNF receptor-associated factor 4;

(3) a DNA encoding the protein of (1);

(4) the DNA of (3) comprising the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3;

(5) a DNA hybridizing with a DNA comprising the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3, and ncoding a protein capable of associating with TNF receptor-associated factor 4;

(6) a vector comprising the DNA of (3);

(7) a transformant harboring the vector of (6);

(8) a method for preparing the protein of (1) comprising a step of culturing the transformant of (7);

(9) an antisense DNA for the DNA of (4) or a portion thereof;

(10) an antibody binding to the protein of (1) or (2); and

(11) a method for screening a compound capable of inhibiting the association between TNF receptor associated factor 4 and the associated factor thereof, comprising

(a) contacting a candidate substance and TNF receptor associated factor 4 with the protein of (1) or (2), and

(b) determining the amount of the protein of (1) which has associated and/or not associated with TNF receptor associated factor 4.

Brief Description of the Drawings

Fig. 1 shows the results of genomic southern blot analysis of various cell lines using TFAF1 cDNA as the probe. The DNA of the HUC-Fm or IMR32 cell line was digested with restriction enzymes BamHI, EcoRI, and HindIII. Electrophoresis of the fragmented DNA was performed in 0.8 % 0.5X TBE agarose gel, and the products were transferred to Hybond-N+ (Amersham). The ^{32}P labelled TFAF1 cDNA fragment was hybridized as the probe.

Fig. 2 shows the results of northern blot analysis of various human tumor cell lines using TFAF1 cDNA as the probe. The total RNA of various human tumor cell lines was isolat d by acid guanidium - phenol chloroform methods. Electrophoresis of the RNA was performed

in 1% agarose gel contaning 0.5% MOPS and 2.2M formaldehyde, and the products were transferred to Hybond-N+ (Amersham). The ^{32}P labelled TFAF1 cDNA fragment was hybridized as the probe.

Fig. 3 shows the results of western blot analysis of various cell lines with mouse anti-TFAF1 antiserum. The lysates were prepared and proteins separated by SDS-PAGE on 12.5% gel. Western blotting was performed with mouse anti-TFAF1 antiserum. Positions of molecular weight standards (in kilodaltons) are shown on the left.

Fig. 4 shows the results of western blot analysis of various cell lines with rabbit anti-TFAF2 polyclonal antibody. The lysates were prepared and proteins separated by SDS-PAGE on 10% gel. Western blotting was performed with rabbit anti-TFAF2 IgG. Positions of molecular weight standards (in kilodaltons) are shown on the left.

Fig. 5 shows the results of pull-down assay (*in vitro*) with TFAFs and TRAF4. TRAF4 was expressed as glutathion S-transferase (GST) fusion protein of pGEX vector (Pharmacia). GST-TRAF4 was purified using Glutathion Sepharose 4B (Pharmacia). ^{35}S labelled TFAFs were generated with the TNT T7 Coupled Reticurocyte Lysate System (Promega) and the various TFAFs expression constructs in pcDNA3-HA.

Fig. 6 shows the results of pull down assay (*in vivo*) with TFAFs and TRAF4. 293T cells were transiently transfected with pcDNA3-MYC-TRAF4 and the indicated pcDNA3-HA-tagged construct. After 48hr, extracts were prepared and immunoprecipitated with monoclonal antibody to MYC epitope. Copr cipitating HA-tagged-TFAFs were detected by immunoblot analysis using the anti-HA monoclonal antibody

(12CA5).

Fig. 7 shows the results of pull down assay (*in vivo*) with TFAF1 and TRAFs. 293T cells were transiently transfected with pcDNA3-MYC-TFAF1 and the indicated pcDNA3-HA-tagged TRAFs constructs. After 48hr, extracts were prepared and immunoprecipitated with polyclonal antibody to TFAF1. Coprecipitating MYC-tagged TRAFs were detected by immunoblot analysis using the anti-MYC monoclonal antibody.

Fig. 8 shows the sequence alignment of TFAF2 with SNX1.

Fig. 9 shows the structure and locations of TRAF4 cDNA and primer used in embodiments.

Detailed Description of the Invention

Amino acid sequences of novel proteins TFAF1 and TFAF2 obtained by the present inventors are shown in SEQ ID NO:2 and SEQ ID NO:4, respectively, nucleotide sequences of DNAs encoding said proteins in SEQ ID NO:1 and SEQ ID NO:3, respectively. These amino acid sequences were assumed based on novel genes with structures determined by screening a human placenta cDNA library using a two-hybrid system. The two-hybrid system is a highly sensitive method for confirming the protein interaction, and has become readily performable these days with a commercial kit. In principle, the system utilizes the cooperation between the DNA binding domain and the transcriptional activation domain in a typical system of transcriptional regulatory factor. In particular, a protein supporting the transcriptional activity has roughly two separated

domains in its structure, one in charge of binding to DNA and the other having the transcriptional activity. Without the cooperative interaction between these two domains, no transcription occurs. In the two-hybrid system, the two domains are separately expressed so that the transcriptional activity is expressed only when a candidate substance associates with its partner; neither the DNA binding domain nor the transcription activating factor alone can express the transcriptional activity. For example, two vectors are constructed; one vector contains a gene encoding a candidate protein (unknown) to be examined for the interaction and the other vector contains a gene encoding its interacting partner protein (known) so that both of the genes are expressed as a fusion protein with the DNA binding domain and the transcription activating factor. Since the expression product of the vector inserted with the gene encoding the partner protein will associate with the desired protein, the vector is thus called a bait. In contrast, a system wherein a marker gene will be expressed by the transcriptional activation factor is previously incorporated into a host (for example, yeast) to be transformed with these two vectors. The DNA binding domain and the transcriptional activation factor fused with their respective proteins are conjugated only when a candidate protein interacts with its partner protein. This allows induction of the marker gene expression. Combinations of interacting proteins can be screened using this marker gene as an index. The present inventors discovered a novel factor and clarified its structure by applying this method to search for substances capable of associating with TRAF4.

Proteins in the present invention include not only those disclosed in SEQ ID NO:2 and SEQ ID NO:4, but also mutants thereof having equivalent biological activities. In other words, the present invention includes all proteins comprising amino acid sequences according to either SEQ ID NO:2 or SEQ ID NO:4, or those having one or more amino acids substituted, deleted or added in amino acid sequences of said proteins and capable of associating with TRAF4. Methods for inducing mutations into the amino acid sequence while maintaining its biological activity are well known. For example, chemical mutagenesis is a known method for producing mutants using random mutagenesis (Myers, R. M., et al., Methods Enzymol., 1987; 155: 501-527). In this method, a base modifying reagent is added to a single stranded DNA of an interesting gene to induce random mutagenesis. With the single-stranded DNA obtained above as a template, double-stranded DNAs are then synthesized by PCR using suitable primers and cloned. Once a clone which gives rise to the expression product having the desired activity is selected from a library of mutant DNAs, it becomes possible to obtain the desired mutant. Futhermore, a target-specific mutant can be prepared by performing PCR with a gene of interest as the template using transformant oligonucleotide primers [Ito, W., et al., Gene 1991, Jun. 15, 102 (1): 67-70]. In addition, mutation is introduced in the amino acid sequence not only by artificial procedures but also in a natural environment. Mutants in the present inv ntion includ those naturally occurring ones so far as they maintain the capability of associating with TRAF4. However, any proteins functionally

capable of associating with TRAF4 equivalent to that of the native TFAFs having amino acid sequences according to SEQ ID NO:2 or SEQ ID NO:4 as the standard may be included. Functionally equivalent substances can be screened, for example, based on whether they act competitively (that is, suppressively) on the association of TRAF4 with proteins having amino acid sequences according to SEQ ID NO:3 or SEQ ID NO:4. More specifically, for example, processes (a) or (a') and (b) described below screen compounds having suppressive activity for the association of TRAF4 with its associated factors.

(a) Simultaneously contacting a candidate substance and TRAF4 with the protein of the present invention, or

(a') Contacting a candidate substance first with TRAF4 and then further with the protein of the present invention, and

(b) Determining the amount of the protein of the present invention which has associated and/or not associated with TRAF4.

Alternatively, combinatorial chemistry may be applied. First, a library of candidate compounds is prepared, then the protein of this invention together with TRAF4 are added to this library. Tracing TRAF4 association with said library results in screening competitive inhibitors (antagonists) for TFAFs. In contrast, tracing TFAFs associating with candidate compounds as the marker results in screening agonists which block the association of TFAFs with TRAF4.

In the screening method of the present invention, the proteins of this invention may be any proteins having the activity domain for associating with TRAF4, not necessarily complete molecules having amino acid sequences described in SEQ ID NO:2 or SEQ ID NO:4. To detect

the association of candidate compounds and proteins of this invention, these proteins are previously modified with detectable molecule labels. The labels may be exemplified by isotope, fluorescent substance, emission substance, enzymatically active substance, etc. In the above-described combinatorial chemistry, a library of candidate compounds which have been immobilized on the solid phase facilitates procedures including the label measurement following the isolation of reaction solution and washing.

Furthermore, these methods are not only useful for screening mutants in the present invention, but also can be used as a general method for screening compounds inhibiting the association of TRAF4 with proteins of this invention. Since compounds screened through this method can controll the signal transduction system wherein TRAF4 is involved, proteins, their antibodies, analogous substances, etc. can be expected to suppress the tumor transformation of cells, especially, breast cancer.

Proteins of the present invention can be obtained by isolating and purifying them from cells expressing TFAF1 or TFAF2. Cells expressing the sought proteins in a high degree should be selected. Since the nucleotide sequence encoding the sought protein is given, persons skilled in the art routinely screen cell lines highly expressing the target gene using a probe based on this sequence. In addition, methods for purifying desired proteins by combining various methods to extract proteins from th cell culture and purify them can be mpirically selected by those skilled in the art. More specifically, various purification methods, including gel filtration,

ion exchange chromatography, reverse phase chromatography, and immuno-affinity chromatography, may be used. Besides purification from naturally occurring materials, proteins of the present invention can be obtained by genetic engineering techniques. For example, based on nucleotide sequences shown in SEQ ID NO:1 and SEQ ID NO:3, the reading region thereof is introduced into an appropriate vector to construct an expression vector. Transfection of a suitable host with this expression vector then allows us to express desired TFAFs as recombinants.

In addition, the present invention provides DNAs encoding proteins of this invention. DNAs produsedaccording to SEQ ID NO:1 or SEQ ID NO:3 disclosed by this invention are novel ones. In a data-base survey, TFAF2 of SEQ ID NO:3 showed a wide range of homology, although weak, with sorting nexin 1 (SNX1). SNX1 has been reported to be a molecule involved in sorting EGF receptors to lysosomes [Kurten, R. C. et al., Science 1996, May 17; 272 (5264): 1008-1010]. However, SNX1 is not meant to predict the structure and function of TFAF1 and TFAF2. For DNAs of the present invention, the desired gene can be obtained by screening a cDNA library using probes designed based on the nucleotide sequence, for example, according to SEQ ID NO:1 or SEQ ID NO:3. In addition, genes of the present invention can be obtained by performing PCR with a cDNA library as the template using primers synthesized based on the nucleotide sequence according to SEQ ID NO:1 or SEQ ID NO:3. Probes and primers can b designed and prepared by the methods known to those skilled in the art. A domain with a sequence 15 to 200mer long, preferably about 20 to 50mer long

and as specific as possible to the desired gene, should be selected to maintain sufficient specificity and reactivity under usual stringent conditions. For primers used in PCR, select sequences near the 5'- and 3'-termini of the segment to be amplified. In this case, the addition of a restriction enzyme recognition sequence to the 5'-side of each primer may facilitate its insertion into vectors. The sequence of SEQ ID NO:1 is about 1.5 kbp long, and that of SEQ ID NO:3, about 2 kbp long. For a sequence of this size, its entire region can be amplified by one PCR with cDNA as the template using one set of primers. A highly sensitive detection of the desired gene can be achieved by identifying amplified products by electrophoresis. Alternatively, expression vectors can be constructed by digesting amplified products with the appropriate restriction enzyme and incorporating digests into vectors.

DNAs of the present invention include not only those constructed with nucleotide sequences as shown in SEQ ID NO:1 and SEQ ID NO:3 but also mutants thereof. Mutants of DNAs of this invention are classified into the following two groups. First, DNAs comprising nucleotide sequences encoding all proteins of the present invention having mutations in the above-described amino acid sequences are just DNA mutants of this invention. More specifically, DNA mutants of this type encoding all mutants having mutations in the amino acid sequences but still maintaining the activity as TFAF1 and TFAF2 (hereafter subtypes of TFAF will be ref rred to as TFAFs as a whole) are included in DNAs of this invention, regardless of whether they are hybridizable with DNAs of SEQ ID NO:1 or SEQ ID NO:3. Since the codon for one amino

acid usually corresponds to multiple sequences of three nucleotides (degeneracy), an astronomical number of sequences is theoretically assumed for the nucleotide sequence of DNA encoding a given amino acid. For this reason, the nucleotide sequence of DNAs of this invention must be specified independently from the complementarity to a specific sequence.

Second, DNAs which are hybridizable with nucleotide sequences of SEQ ID NO:1 or SEQ ID NO:3 and encode proteins maintaining the activity as TFAFs are included in DNAs of the present invention. It is generally accepted that many proteins encoded by nucleotide sequences hybridizable with a specific sequence under stringent conditions often have activities similar to that of the protein encoded by the specific sequence.

DNA sequences of the present invention including mutants thereof can be applied to various usages based on well-known techniques. First, the protein encoded by the DNA can be obtained as a recombinant by applying DNA of the present invention to an appropriate expression system.

Based on DNA of this invention, a probe for detecting genes for TFAFs can be set up routinely. Persons skilled in the art set up a probe for a given sequence. Oligonucleotide comprising the nucleotide sequence of a set up probe can be obtained by chemical synthesis. By suitably labeling that nucleotide, it can then be used for the hybridization assay of various formats.

The present invention provides antibodies recognizing prot ins according to this invention. Antibodies of this inv ntion can be

prepared by immunizing with proteins and fragments thereof obtained by the method described above. For the immunization, immunogens are mixed with an adjuvant such as FCA, and subcutaneously inoculated into an animal in accordance with an appropriate schedule. A high immunological stimulation can be expected when an animal with a TFAF structure that differs as much as possible from that of humans is selected for immunization. Antibodies include not only polyclonal antibodies purified from the sera of immunized animals, but also monoclonal antibodies which can be obtained by cloning antibody generating cells. A method for producing monoclonal antibodies by recovering antibody-generating cells from immunized animal and fusing them with culture cell lines to allow cloning is obvious to those skilled in the art. Antibodies thus obtained can be used for the immunological detection of TFAF according to this invention as well as the purification thereof.

Furthermore, it is possible to humanize the antibody by utilizing the gene in the variable region of the antibody from antibody generating cells recognizing TFAFs derived from an animal of a different species. Specifically, it is possible to produce a chimeric antibody comprising the constant region of human antibody in the variable region of mouse antibody by the genetic recombination technique. Alternatively, there is also a method for obtaining a humanized antibody wherein only a hypervariable region is incorporated into the framework of a human antibody. Since th s humanized antibodies are hardly immuno-r active when administered to humans, they can be used both safely and effectively *in vivo*. Tumors,

including breast cancer, can be diagnosed and treated by administering *in vivo* antibodies recognizing TFAFs. In cancer diagnosis, the location and size of tumor can be detected by administering the antibody of this invention which has been labeled with an isotope, and tracing the antibody recognizing and binding cancer cells. To treat cancer, antibodies of this invention are labeled with appropriate drugs, precursors thereof, enzymes to activate drug precursors, etc. prior to administration.

The present invention was used to clarify structures of TFAF1 and TFAF2. Since the expression of TRAF4, a binding partner of TFAFs, is enhanced in breast cancer cells, the involvement of TFAFs in breast cancer is highly possible. Therefore, cancer can be controlled by administering agonists and antagonists of TFAFs to control the interaction between them. TFAFs proteins provided by this invention are useful for screening these agonists (antagonists).

Furthermore, antisense DNA can be obtained based on the nucleotide sequence of DNA encoding TFAFs provided by this invention. By administering this antisense DNA, the control of TFAFs expression leading to the cancer control can be expected.

Proteins, antibodies recognizing them, or DNAs encoding these proteins provided by this invention will provide many tools for promoting this research.

The following will describe the present invention in more detail with r ference to Example, but the present invention is not to be construed to be limited thereto.

EXAMPLE

1. TRAF4 cloning by PCR (Fig. 9)

1.1 Preparation of primers

In order to isolate the C-terminal domain of human TRAF4 gene using PCR, the following two primers were prepared:

5' primer [CART1 F2-Bam (26mer)]

5'-ACG GAT CCT GCC CTA AGC TGG CAA TG-3'

* Two bases (AC) at the 5' terminus are for smooth treatment with restriction enzyme.

The sequence from the third base to the eighth base at the 5' terminus (GGATCC) is the restriction enzyme BamHI site.

3' primer [CART1 R1-Xho (26mer)]

5'-TGC TCG AGC CTG CAC TCA GCT GAG GA-3'

* Two bases (TG) at the 5' terminus are for smooth treatment with restriction enzyme.

The sequence from the third base to the eighth base at the 5' terminus (CTCGAG) is the restriction enzyme XhoI site.

1.2 PCR

A genomic DNA of Jurkat cells derived from human T cells was used as the template for amplifying the C-terminal region of human TRAF4 gene by PCR. The genomic DNA was extracted and purified from Jurkat cells using proteinase K and phenol. PCR conditions are as follows:

a) 94°C, 5 min;

b) 1 cycle of 94°C, 1 min, 57°C, 5 min, and 72°C, 2 min;

c) 35 cycles of 94°C, 2.5 min, 65°C, 1min and 72°C, 1 min; and

d) 72°C, 10 min.

Taq polymerase was used for the reaction.

After PCR, amplified DNAs were identified by 1% agarose gel electrophoresis. After electrophoresed bands were identified, the remaining reaction solution was treated with restriction enzymes BamHI and XhoI. This treatment cleaved the restriction enzyme sites at both terminal regions of amplified PCR products to form termini with cohesive ends. After treatment with these restriction enzymes, amplified fragments were electrophoresed on 1% agarose gel, excised from the gel, and purified by the glass matrix method (BIOTECH, GeneClean).

1.3 Cloning of PCR products into vector

The pAS2-1 vector is a bait vector used in a CLONTECH MATCHMAKER Two-Hybrid System 2, and inserted with the gene segment used as a bait into the multi-cloning site (MCS) located downstream from the sequence encoding GAL4-DNA-BD (binding domain of the GAL4 protein).

In order to match the pAS2-1 vector and the translation frame of the PCR product, the MCS of the pAS2-1 vector was modified. After pAS2-1 was cleaved with a restriction enzyme NdeI, bases were added to the 3'-staggered end to form blunt ends using T4 DNA polymerase, and the vector was self-ligated using T4 DNA ligase to form the pAS2-1Δ NdeI vector. Fragments obtained by treating the pAS2-1Δ NdeI vector with restriction enzymes BamHI and XhoI were purified by the same method used for PCR products. Purified PCR products and fragemnts of the pAS2-1Δ NdeI vector were ligated. First, purified PCR products and the pAS2-1Δ NdeI vector were mixed in a molar ratio of 1 then reacted using T4 DNA liagase at 16°C for 1 h.

A ligation reaction solution containing plasmid DNA was added to *Escherichia coli* DH5α which was made competent by the rubidium chloride method. The mixture was gently stirred, allowed to stand on ice for 30 min, heat-shocked in a warm water bath at 42°C for 30 s, and allowed to stand on ice again for 2 min. An SOC medium was added to this mixture, and the resulting mixture was allowed to stand at 37°C for 1 h, spread onto an LB plate containing ampicillin (50 ng/ml), and incubated at 37°C overnight.

Multiple colonies were harvested from the plates, and cultured in a LB-ampicillin medium at 37°C overnight. The plasmid DNA was recovered from the cultured *E. coli* cells using the alkaline method. Recovered DNA was cleaved with restriction enzymes BamHI and XhoI, and the insertion of PCR products into the vector was confirmed by electrophoresis on agarose gel.

Recovered DNAs were purified by the polyethylene glycol sedimentation method, and PCR products within the vector were identified with a fluorescence sequencer (Perkin-Elmer, ABI Business Section) based on Sanger's method. This procedure confirmed that the insertion of C terminal fragments of human TRAF4 gene into the pAS2-1Δ NdeI vector yielded DNA of pAS2-1Δ NdeI-hTRAF4Cter.

2. Two-Hybrid Screening

A MATCHMAKER Two-Hybrid System 2 (CLONTECH) was used for the two-hybrid screening.

2.1 Purification of a library DNA us d as prey

A Human Plac nta MATCHMAKER cDNA Library purchased from CLONTECH was used as a library for prey. This library was prepar d using the

pAC2 vector, and the MCS is located downstream of the sequence encoding GAL4-AD (activation domain of the GAL4 protein). cDNA fragments are inserted into this MCS. Bacterial cells inserted with this library were cultured in the LB ampicillin medium, and the plasmid DNA contained in bacteria was recovered and purified by the polyethylene glycol sedimentation method.

2.2 Confirmation of no β-gal actiivty with a bait alone

Two-hybrid screening is based on the principle that the HIS3 gene and the lacZ gene having the GAL4 promoter upstream are expressed when a bait GAL4-DNA-AD fusion protein and a prey GAL4-AD fusion protein are associated. Therefore, if the transcriprtion from the GAL4 promoter is initiated with the bait alone, the two-hybrid screening is not established. Therefore, it is necessary to confirm that the lacZ gene is not expressed with the bait alone, that is, there is no β-gal activity.

The pAS2-1Δ NdeI-hTRAF4Cter plasmid DNA and the herring testes carrier DNA were added to yeast Y190 made competent by the lithium acetate method. A polyethylene glycol-lithium acetate solution (40% polyethylene glycol 4000, 0.1 M lithium acetate, and 1X TE buffer) was then added, and the mixture was cultured on a shaker at 30°C for 30 min. DMSO was added to this culture to achieve a final concentration of 10%, and the mixture was heat-shocked in a warm water bath at 42°C for 15 min, quickly cooled on ice, and centrifuged at 14 Krpm for 5 s. The pellet was suspend d in 1X TE buffer, spread onto a SD/-Trp plate, and incubated at 30°C for 3 days.

Colonies obtained by transformation were harvested and cultured

in the SD/-Trp medium at 30°C for 3 days. Yeast was collected by centrifugation and suspended in an extraction buffer. [A solution containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 300 mM NaCl, 0.5% NP-40, and 5% glycerol was prepared, sterilized, and stored at 4°C. Just prior to use, 3 mM DTT, 1 mM PMSF, and 1 μg/ml pepstatin A were added to this solution to the indicated concentrations.] Yeast was disrupted with glass beads in a Vortex mixer, and centrifuged to recover the supernatant.

A sample buffer for SDS-PAGE was added to this supernatant, and the mixture was boiled, vortexed, centrifuged to remove debris, and subjected to SDS-PAGE. After electrophoresis, proteins were blotted to a PVDF membrane by the semi-dry method. The membrane blotted with proteins was soaked in a blocking buffer (5% skim milk and 0.1% NaN_3 in TTBS buffer) and subjected to immuno-detection using anti-GAl4 monoclonal antibody (CLONTECH) to confirm the expression of a fusion protein between GAL4-DNA-AD and the C terminus of TRAF4.

The plate with growing colonies obtained by transformation was covered with a sterilized nylon transfer membrane (Amersham, Hybond-N+) to transfer clonies to the membrane. This membrane was soaked in liquid nitrogen for 10 s, then returned to room temperature. With the side with colonies adhering faced up, the membrane was placed on a filter paper which had been soaked in a Z-buffer/X-gal solution [100 ml Z buffer (containing 16.1 g/l Na_2HPO_4-$7H_2O$, 5.50 g/l NaH_2PO_4-H_2O, 0.75 g/l KCl and 0.246 g/l $MgSO_4$-$7H_2O$, adjusted to pH 7.0), 0.27 ml mercaptoethanol, 1.67 ml X-gal solution (20 mg/ml X-gal in DMSA)], and allowed to stand at 30°C for 1 h. If the β-gal activity was

expressed with the bait alone, the filter paper will turn blue. However, it did not with pAS2-1Δ NdeI-hTRAF4Cter. In other words, pAS2-1Δ NdeI-hTRAF4Cter alone does not initiate the translation from the GAL4 promoter, confirming the possibility of using pAS2-1Δ NdeI-hTRAF4Cter as a bait.

2.3 Screening

Yeast Y190 which had been made competent by the lithium acetate method and tranformed with pAS2-1Δ NdeI-hTRAF4Cter was further transformed with a purified prey cDNA library of the pACT2 vector. Yeast was streaked on a SD/-Trp/-Leu/-His/+3-AT plate and cultured at 30℃ for 7 days. By this procedure, only yeast which becomes His+ wherein bait and prey are associated to express the His3 gene located downstream of the GAL4 promoter forms colonies. In order to examine whether bait and prey are actually associated to express a gene located downstream of the GAL4 promoter, the inventors investigated whether the lacZ gene, another gene located downstream of the GAL4 promoter, was expressed, that is, whether the yeast had β-gal activity. By measuring the β-gal activity using the colony lift filter assay, they confirmed that a clone which was His+ and had the β-gal activity was obtained.

2.4 Sequencing of prey

In order to examine the nucleotide sequence of the cloned prey obtained by screening, DNAs were recovered from yeast and transferred into *E. coli*. His+ and β-gal active clones were scraped from the plate, and cultured in a SD/-Leu medium overnight. Yeast was coll cted, dissolved in a SZB solution [1 M sorbitol, 0.1 M sodium citrate, 0.06

M EDTA, 0.8% β-mercaptoethanol and 0.6 g/l ZYNOLASE-100T (Seikagaku Kogyo)], and allowed to stand at 30°C for 30 min. The yeast was then completely dissolved in a SDS-TE solution [2% SDS, 0.1 M Tris-HCl (pH 8.0) and 10 mM EDTA]. To this was added potassium acetate to a final concentration of 3.3 M. The resulting suspension was then centrifuged to obtain the supernatant. Ammonium acetate was added to the supernatant thus obtained to a final concentration of 3.3 M, and DNA was suspended using isopropanol. After the supernatant was completely removed, DNA was washed with 80% ethanol, air-dried, and dissolved in sterilized water.

E. coli HB101 made competent with HEPES-NaOH for electroporation was electro-transformed with the plasmid DNA recovered from yeast. A Gene Pulser and a Cubette (BIO-RAD) were used for the electroporation. After the electroporation, an ice-cold SOC medium was added to the bacterial suspension, and the mixure was cultured for recovery on a shaker at 37°C for 1 h. *E. coli* was washed with 1X M9 salts, spread onto a -Leu plate (an M9 plate containing a -Leu dropout solution consisting of 50 μg/ml ampicillin, 40 μg/ml proline and 1 mM thiamin) and incubated at 37°C overnight. Since *E. coli* HB101 has a leuB mutation, only a prey plasmid DNA (from the bait DNA of the pAS2-1 vector and prey DNA of the pACT2 vector obtained from yeast) having the LEU gene capable of filling out the leuB mutation can transform *E. coli* HB101, forming colonies.

Plasmid DNA in *E. coli* HB101 was recovered by extracting it by the alkaline method and deproteinizing it with phenol-chloroform. E. coli DH5α was transformed with the plasmid DNA thus obtained. The

inventors succeeded in transferring the plasmid DNA into E. coli DH5α, from which said plasmid DNA can be recovered in a large quantity.

Plasmid DNA of the pACT2 vector in E. coli DH5α was recovered by the alkaline method and purified by the polyethylene glycol sedimentation method. The gene nucleotide sequences in the vector were identified using a fluorescence sequencer based on the Sanger's method.

Through the above-described screening, eleven genes were finally identified on the plasmid, and their nucleotide sequences were determined, resulting in a novel gene TFAF1 (#127 and #288), a novel gene TFAF2 (#293T and #315), dystrophin Cter (#187, #233, #463 and #479), and TRAF4 (#353, #372 and #404) (clone numbers indicated in parentheses). Nucleotide sequences of TFAF1 and TFAF2 are shown in SEQ ID NO:1 and SEQ ID NO:3, respectively. Furthermore, there was a clone comprising the full length of the TRAF4 gene, indicating the homodimer formation of TRAF4.

2.5 Confirmation by re-transformation

By measuring the β-gal activity after yeast Y190 was transformed with purified DNAs of pACT2-TFAF1 and pACT2-TFAF2, the inventors confirmed that a fusion protein of GAL4-AD protein with TFAF1 or that with TFAF2 alone did not initiate the transcription from the GAL4 promoter. Also, by measuring the β-gal activity in yeast Y190 which had been transformed with pAS2-1Δ NdeI-hTRAF4Cter and pACT2-TFAF1 or pACT2-TFAF2, they confirmed that the transcription from the GAL4 promoter occurred, that is, and that hTRAF4Ct r and TFAF1 or TFAF2 were associated in the yeast nucleus.

3. Genomic Southern Blot Analysis (TFAF1) (Fig. 1)

Genomic Southern Blot Analysis was performed using the genomic DNA of the HUC-Fm cell line derived from human umbilical cord fibroblast, IMR-32 cell line derived from human fibroblastoma, and WR19L cell line derived from mouse lymphoma as the sample and TFAF1 segment as the probe.

3.1 Recovery of genomic DNA

Each cell line cultured in DME medium (+ 10% FCS) was collected and suspended in a DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 10 mM EDTA]. To this suspension were added SDS to a final concentration of 0.1% and proteinase K to a final concentration of 100 μg/ml. The mixture was then gently stirred, occasionally mixed at 55°C for 1 h, and treated on a rotary shaker at 37°C overnight. An equal volume of phenol was added to this mixture, and the mixture was gently mixed on a shaker at room temperature for 20 min then centrifuged. An equal volume of phenol/chlroform/isoamyl alcohol was added to the upper layer (aqueous phase), and the mixture was gently mixed on a shaker at room temperature for 20 min. After centrifugation, the upper layer (aqueous phase) was exhaustively treated with phenol/chloroform/isoamyl alcohol until proteins were completely removed. The deproteinized upper layer (aqueous phase) was gently mixed with ethanol. DNA appearing in the interface was entwined with a glass rod, washed with 70% ethanol, and air-dried. The DNA together with the glass rod was soaked in a TE buffer, and allowed to stand overnight to dissolve DNA.

3.2 Electrophoresis

Genomic DNA thus recocvered was treated with restriction enzymes BamHI, EcoRI and HindIII. Restriction enzymes were used at relatively high concentrations and reacted overnight so that DNA was surely fragmented. Fragmented DNAs were electrophoresed in 0.8% 0.5X TBE agarose gel at low voltage overnight. After electrophoresis, gel was soaked in a solution of ethidium bromide (1 μg/ml) to stain DNA. After locations of size markers on the gel were confirmed by photography, the gel was UV irradiated to sectionalize DNA fragments for easy transfer. After the sectionalization, the gel was soaked in an alkaline transfer solution (0.1 M NaCl and 0.1 N NaOH) to denature DNA. DNA in the gel was transferred onto a nylon transfer membrane (Amersham, Hybond-N+) by the capillary transfer method using the alkaline transfer solution. The membrane having DNA transferred was neutralized by soaking it in 2X SSC and air-dried. The transferred DNA was then cross-linked to the membrane using a UV cross-linker (Stratagene, Stratalinker 1800).

3.3 Preparation of probe

A DNA fragment of about 1.6 Kbp obtained by cleaving pACT2-TFAF1 with restriction enzymes BamHI and XhoI was re-cloned into the pcDNA3-HA-epitope-tagged vector (pcDNAha; prepared by modifying pcDNA3 (Initrogen)) to form pcDNAha-TFAF1. A fragment of about 700 bp obtained from this pcDNAha-TFAF1 by cleaving the cloning site at the 5' terminus of cDNA of TFAF1 with restriction enzyme SalI and the internal region of cDNA of TFAF1 with restriction nzyme HindIII was recovered by isolation using electrophoresis and the purification using the glass matrix method. [^{32}P]labelled probes were prepared

with the DNA fragment thus obtained as the template, using a DNA random labelling kit (Amersham, Rediprime DNA labelling system) and [α-^{32}P]deoxy-CTP (ICN) and purified using a spin column (Pharmacia, ProbeQuant G-50 Micro Column).

3.4 Hybridization

Hybridization was performed using a hybri-bottle and a hybri-oven (Taitech). A membrane having DNA cross-linked was prehybridized in a hybridization buffer (10% PEG6000, 1.5% SSPE and 7% SDS) at 65°C for 1 h. A [^{32}P]labelled probe was boiled, quickly cooled, diluted with the hybridization buffer prewarmed to 65°C, and replaced with the solution used for prehybridization. Hybridization was performed at 65°C overnight.

The membrane was washed with a solution containing 2X SSC and 0.1% SSC to remove the hybridization buffer. It was further washed with a solution containing 0.1X SSC and 0.1% SDS at 65°C followed by a solution containing 0.1X SSC and 0.1%SDS at room temperature. The washing extent was occasionally checked using a survey meter. Washing solutions were exchanged several times until the count in the membrane became low. When the count became low, the excessive water in the membrane was absorbed with 3MM filter paper (Whatman). The membrane was then mounted on a pasteboard and wrapped in a plastic sheet.

The pasteboard with the membrane wrapped in a plastic sheet was placed in a cassette for autoradiography, and a film was placed on the membrane mounting side. The cassette was placed in a -80°C freezer overnight and returned to ro m temperature; the film was then

developed. The cassette and film used were a Hypercasette and a Hyperfilm-MP (Amersham). Results are shown in Fig. 1. It is evident that there is one TFAF1 present in the genome, and no sequentially homologous gene.

4. Northern Blot Analysis (TFAF1) (Fig. 2)

Northern Blot Analysis was performed using the following cell lines as the total RNA sample and the same TFAF1 segment as in 3 as the probe:

SUDHL6 cell line derived from human follicular lymphoma,

ZR75-1 cell line derived from human epithelial breast cancer,

KATOIII cell line drived from human gastric cancer,

HepG2 cell line derived from human liver cancer,

Raji cell line derived from human Burkitt lymphoma,

MOLT-4 cell line derived from human acute lymphocytic leukemia,

MKN45 cell line derived from human gastric cancer,

IMR-32 cell line derived from human fibroblastoma,

KB3-1 cell line derived from human oral epitheloid cancer,

HL60 cell line derived from human acute promyelogenic leukemia, and

Jurkat cell line derived from human T cell acute lymphoblastoid leukemia.

4.1 Recovery of total RNA

Total RNA was isolated by the acid guanidium-phenol chloroform method (NipponGene, Isogen). Each cell line cultured in DME medium (+ 10% FCS) was collected, and Isogen was added. Cells were susp nded by pipetting, fully mixed with added chloroform, and centrifuged.

The total RNA contained in the supernatant (aqueous phace) was recovered by ethanol sedimentation. After being washed with 70% ethanol, the total RNA was dissolved in RNase-free diethyl pyrocarbonate (DEPC) water.

4.2 Electrophoresis

Total RNA thus recovered was electrophoresed using a gel comprising 1% agarose, 0.5% MOPS and 2.2 M formaldehyde. After electrophoresis, the size marker section was excised and stained with ethidium bromide to confirm the locations of size markers. The total RNA section was soaked in 50 mM NaOH to alter the conformation of RNA and neutralized with 200 mM sodium acetate. RNA in the gel was then transferred to a nylon transfer membrane (Amersham, Hybond-N+) by the capillary transfer method. With the surface having RNA transferred faced up, the membrane was allowed to stand on a filter paper previously soaked in 50 mM NaOH to alkali-immobilize RNA on the membrane. After neutralizing with 2 x SSC, the membrane was completley dried in an oven at 80°C.

4.3 Hybridization

Hybridization was carried out using a hybri-bag. The membrane having RNA transferred was prehybridized in a hybridization buffer (CLONTECH, ExpressHyb Hybridization Solution) at 68°C for 30 min. A [^{32}P]-labelled probe (the same as prepared in 3.3) was boiled, quickly cooled, diluted with the hybridization buffer warmed to 68°C, and replaced with the solution used for prehybridization. Hybridization was performed at 68°C for 1 h.

The membrane was rinsed with a washing solution containing 2 x

SSC and 0.1% SDS at 65°C to remove the hybridization buffer. It was then washed in a solution containing 0.1 x SSC and 0.1% SDS at room temperature. Washing extent was occasionally confirmed with a survey meter. Washing solutions were changed several times until the count in the membrane became low. When the count became low, excessive water of the membrane was absorbed with a 3MM filter paper (Whatman). The membrane was then mounted on a pasteboard and wrapped with a plastic sheet.

The pasteboard with the membrane wrapped in a plastic sheet was placed in a cassette for autoradiogram, and a film was placed on the membrane mounting side. The cassette was placed in a -80°Cfreezer for 3 days then returned to the room temperature, after which the film was developed. Results are shown in Fig. 2. It is evident that TFAF1 is transcripted as mRNA of about 1600 bp in many cell lines.

5. Preparation of Antibodies

5.1 Preparation of anti-TFAF1 antibodies (mouse anti-serum)

In order to prepare antibodies against the TFAF1 protein, its fusion protein with glutathione-S-transferase (GST) protein was purified, and anti-TFAF1 mouse anti-seerum was obtained by immunization with this fusion protein as the antigen.

First, the TFAF1 fragment was re-cloned from pACT2-TFAF1 into the pGEX vector (Pharmacia) to construct pGEX-TFAF1. After E. coli (DH5 α) which had been transformed with this pGEX-TFAF1 was cultured in an LB-ampicillin medium at 37°C overnight, the culture was added to a fresh LB-ampicillin medium to dilute it several tens of times. It was then further incubated at 37°C for several hours. When the

turbidity reached 0.6 to 1.0, IPTG was added at a final concentration of 1 mM to induce the expression of GST-TFAF1 fusion protein. The culture was then further incubated at 30°C for several hours. After the incubation, bacterial cells were collected by centrifugation, and a portion thereof was subjected to SDS-PAGE and stained with Coomassie blue to confirm the expression of GST-TFAF1 fusion protein.

The remaining cells were fully suspended in PBS containing 1% Tween 20 and completely disrupted by ultrasonication. A soluble GST-TFAF1 fusion protein contained in the supernatant obtained by centrifuging the sonicate was loaded onto a GSH-Sepharose 4B column (Pharmacia) to absorb said fusion protein by binding between GST and GSH. After the column was thoroughly washed with WE buffer [10 mM β-mercaptoethanol, 2 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.5)], the GST-TFAF1 fusion protein was eluted with G buffer [10 mM GSH and 50 mM Tris-HCl (pH 9.6)]. An aliquot of the eluate was subjected to SDS-PAGE and stained with Coomassie blue to confirm the purification of GST-TFAF1 fusion protein. The eluate was condensed with PBS containing 50% glycerol, and the buffer was changed.

Using a 1 ml-syringe and 21-gauge needle, the purified GST-TFAF1 fusion protein and Freund's complete adjuvant were mixed to a complete emulsion in a 1.5 ml tube and injected into the peritoneal cavity of a mouse. After repeating this procedure for 5 weeks, the blood was collected and centrifuged to recover the anti-serum in the supernatant, that is, the anti-TFAF1 mouse serum.

Reactivity of the anti-serum was examined by the ELISA method to confirm its reaction with TFAF1 protein.

5.2 Preparation of anti-TFAF1 polyclonal antibodies and anti-TFAF2 polyclonal antibodies (rabbit antibody)

In order to prepare rabbit antibody for TFAF1 and TFAF2 (hereafter may be referred to as TFAF1/2, including both in one group), the fusion protein with glutathione-S-transferase (GST) was purified, and a rabbit was immunized with said purified protein to recover anti-TFAF1/2 anti-serum. From this anti-serum, anti-TFAF1/2 antibody capable of specifically binding to TFAF1/2 protein was purified by affinity purification using TFAF1/2 protein with a 6His-tag attached.

First, pGEX-TFAF2 was constructed and GST-TFAF2 fusion protein was purified by similar procedures as described in [5.1 preparation of anti-TFAF1 antibodies (mouse anti-serum)].

Using a 1 ml-syringe and 21-gauge needle, purified GST-TFAF1 fusion protein (or GST-TFAF2 fusion protein) and Freund's complete adjuvant were mixed to a complete emulsion in a 1.5 ml tube, and injected subcutaneously into a rabbit. After repeating this procedure every week for three times, a preliminary blood sample was withdrawn, centrifuged to recover the anti-serum in the supernatant, and checked for its reactivity by ELISA. This confirmed that the serum was reactive with TFAF1 protein and TFAF2 protein. Thereafter, immunization, actual blood collection and no-treatment were repeated every other week.

6His-TFAF1/2 protein was purified in order to purify the antibody from the anti-serum obtained using GST-TFAF1/2 as antigen by affinity chromatography. TFAF1/2 fragments were re-cloned from pGEX-TFAF1

and pGEX-TFAF2 into the pET vector (Invitrogen) to construct pET-TFAF1 and pET-TFAF2. Similarly as described for the expression of GST-TFAF1 fusion protein in [5.1 Preparation of anti-TFAF1 antibodies (mouse anti-serum)], pET-TFAF1 and pET-TFAF2 were expressed in *E. coli* [AD494 (DE3)], and cells were harvested. Cells were suspended in Ni-Agarose binding buffer [5 mM immidazole, 0.5 M NaCl and 20 mM Tris-HCl (pH 7.9)] containing 0.1% NP-40, and completely disrupted by ultrasonication. Soluble 6His-TFAF1/2 protein contained in the supernatant obtained by centrifuging sonicates was loaded onto a Ni-Agarose column charged with Ni+ by NiSO4, and absorbed into the column by binding of 6His to Ni+. After the column was thoroughly washed with Ni-Agarose binding buffer, the column was eluted with a linear gradient concentration of immidazole in the elution buffer (10 mM-1000 mM immidazole, 0.5 M NaCl and 20 mM Tris-HCl) into several fractions. An aliquot of each fraction was subjected to SDS-PAGE and stained with Coomassie blue to confirm and recover fractions containing 6His-TFAF1/2 protein. The eluate was condensed with PBS containing 50% glycerol, and the buffer was changed.

CNBr-activated Sepharose 4B having the active group to react with the primary amino group in proteins (Pharmacia) was gelated by swelling and mixed with purified 6His-TFAF1 or 6His-TFAF2 proteins overnight. The mixture was transferred to a column, thoroughly wash d with PBS, and treated with 1 M Tris-HCl to block active groups; the buffer was then changed to PBS. Similarly, an E. coli protein affinity column was prepared using E. coli suspended in PBS containing

1% Triton X100 to absorb E. coli-reactive antibodies.

Anti-TFAF1- or anti-TFAF2-antiserum was diluted twice with PBS containing 0.2% Triton X100 and applied to a 6His-TFAF1 or 6His-TFAF2 protein affinity column pre-equibrated with PBS. The column was washed with PBS until O.D.280 of washings through the column became nil then eluted with an elution buffer [0.17 M glycine-HCl (pH 2.3) and 10% glycerol] into a few fractions, to which pH adjusting buffer [1 M Trip-HCl (pH 9.6)] was immediately added. O.D.280 of each fraction was measured, and fractions containing antibodies were collected. Antibody solutions thus obtained were treated in an *E. coli* protein affinity column to absorb antibodies reacting with *E. coli*. Non-absorbed fractions were collected and dialyzed against PBS.

Antibodies thus obtained were examined for their reactivity by the ELISA method and Western blot analysis to confirm that they react with TFAF1 and TFAF2 proteins. Although anti-TFAF1 polyclonal antibody thus obtained did not specifically and strongly react with the endogenous TFAF1, it was confirmed that this antibody can be used for immunoprecipitation of forcibly expressed TFAF1. In contrast, it was confirmed that anti-TFAF2 polyclonal antibody specifically and strongly reacts with the endogenous TFAF2.

6. Western Blot Analysis (Figs. 3 and 4)

6.1 Western blot analysis of TFAF1

Western blot analysis was performed using th following cell lines as the sample and anti-TFAF1 mous anti-serum:

Jurkat cell line derived from human T cell acute lymphoblastoid

leukemia,

HeLa cell line derived from human cervical cancer,

293T cell line derived from human fetal renal cell,

HUC-Fm cell line derived from human umbilical cord fibroblast, and

293T Cell line which had been transfected with pcDNAha-TFAF1 to transiently express TFAF1 protein.

Each cell line cultured in DME medium (+ 10% FCS) was collected, suspended in E1A buffer [50 mM HEPES (pH 7.4), 250 mM NaCl, 0.1% NP-40, 5 mM EDTA and 10% glycerol], allowed to stand in ice, and centrifuged to recover the supernatant containing soluble proteins. A sample buffer for SDS-PAGE was added to an aliquot of this supernatant, and the mixture was boiled then subjected to SDS-PAGE. After electrophoresis, proteins were blotted onto a PVDF membrane by semidrying. The membrane with blotted proteins was soaked in a blocking buffer (5% skim milk and 0.1% NaN_3 in TTBS buffer) and subjected to immunodetection with anti-TFAF1 mouse serum to confirm the presence of endogenous TFAF1 protein in each culture cell line. The results shown in Fig. 3 reveal that TFAF1 is a protein with a molecular weight of about 30 KD, and that TFAF1 is expressed in many cell lines.

6.2 Western blot analysis of TFAF2

Western blot analysis was performed using the following cell lines as the sample and anti-TFAF2 mouse antibody;

Jurkat cell line derived from human T cell acute lymphoblastoid leukemia,

Raji cell line derived from human Burkitt lymphoma,

HL60 cell line derived from human acute promyelogenic leukemia,

ZR75-1 cell line derived from human epithelial breast cancer,

MOLT-4 cell line derived from human acute lymphocytic leukemia,

HeLa cell line derived from human cervical cancer,

HUC-Fm cell line derived from human umbilical cord fibroblast,

NIH-3T3 cell line derived from mouse embryo,

CHO cell line derived from hamster ovary fibroblast,

293T cell line derived from human fetal renal cell, and

293T Cell line which had been transfected with pcDNAha-TFAF2 to transiently express TFAF2 protein.

Sample proteins were prepared similarly as in the case of TFAF1. After SDS-PAGE, blot and blocking were performed, and they were subjected to immunodetection with anti-TFAF2 rabbit polyclonal antibody (purified by immunoaffinity chromatography) to confirm the presence of endogenous TFAF2 protein in each culture cell line. The results shown in Fig. 4 demonstrate that TFAF2 is a protein with a molecular weight of about 45 KD.

7. Comprehensive Experiments (Figs. 5, 6 and 7)

Two-hybrid screening is a method for screening proteins based on their mutual association under specific physiological conditions, that is, in the yeast nucleus. For the novel gene TFAF1 obtained in this invention, it is necessary to examine the association of its expression product both under specific conditions, that is, in the yeast nucl us, and under more physiological conditions. Therefore, the association between TRAF4 and TFAF1 *in vitro* was confirmed by

pull-down assay; the association *in vivo*, by an immunoprecipitation experiment; and the specificity of association between TRAF1-6 and TFAF1 *in vivo*, by an immunoprecipitation experiment.

7.1 Association between TRAF4 and TFAFs (*in vitro* pull-down assay)

The association between GST-TFAFs fusion protein(s), ^{35}S-labelled TFAF1/2 obtained by *in vitro* translation and TRAF4 protein was examined by *in vitro* pull-down assay.

In vitro translation was performed using a TNT T7 Coupled Reticulocyte Lysate System (Promega). DNAs prepared by recloning each gene fragment in the pACT2 vector obtained by two-hybrid screening into the pcDNAha vector (pcDNAha-TFAF1, pcDNAha-TFAF2, and pcDNAha-TRAF4) were used. Reaction solutions were prepared and incubated at 30°C according to instructions supplied with the kit. *In vitro* translation was confirmed by SDS-PAGE analysis of aliquots of the reaction solution.

From pACT2-TRAF4 obtained by two-hybrid screening, a TRAF4 fragment was re-cloned into a pGEX vector to construct pGEX-TRAF4. Using E. coli (DH5α) transformed with pGEX-TRAF4, GST-TRAF4 was purified as in the case of immunogen preparation. In the purification process, when GSH-Sepharose 4B and GST-TRAF4 were combined, they were mixed with a binding buffer [50 mM Tris-HCl (pH 8.0), 0.1% NP-40, 2 mM EDTA, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM DTT, 5% glycerol and 150 mM NaCl] containing 1% BSA for blocking, then washed with the binding buffer containing no BSA to make a 50% susp nsion. A 50% suspension of GSH-Sepharose 4B-GST for pre-clearing was similarly prepared. Pre-clearing was performed in order

to remove proteins binding to GSH-Sepharose 4B and GST contained in *in vitro* translation products. *In vitro* translation products were dissolved in the binding buffer, mixed with a 50% suspension of GSH-Sepharose 4B-GST, and proteins non-specifically binding to GSH-Sepharose 4B and GST were sedimented by centrifugation. The supernatant thus obtained was used as the sample in pull-down assays.

A 50% suspension of GSH-Sepharose 4B-GST-TRAF4 was mixed with *in vitro* translation products contained in the supernatant after pre-clearing. GSH-Sepharose 4B-GST-TRAF4 and *in vitro* translation products binding thereto were centrifuged and washed with a binding buffer containing no BSA. Translation products were dissolved in a sample buffer and subjected to SDS-PAGE. Gel was fixed, enhanced, and air-dried, and ^{35}S-labelled *in vitro* translation products were detected by autoradiography. Results shown in Fig. 5 demonstrate that TRAF4 associates with TFAF1/2 *in vitro* and that TRAF4 forms a homodimer *in vitro*.

7.2 Association of TRAF4 and TFAFs (*in vivo* immunoprecipitation)

pCMV6myc-TRAF4, which was constructed by re-cloning TRAF4 into myc-epitope-tag vector pCMV6myc, and pcDNAha-TFAF1, pcDNAha-TFAF2 or pcDNAha-TRAF4 were co-transfected into the 293T cell line derived from human fetal renal cell. Their intracellular associations when they are forcibly expressed were confirmed by an *in vivo* immunoprecipitation experiment. Anti-MYC monoclonal antibody (9E10) was used for immunoprecipitation, and anti-HA monoclonal antibody was used for immunodetection (Boehring r, Mannheim).

Trans IT (PanVera) was used for co-transfection based on the

liposome method. DNA and a Trans IT solution were mixed in the DME medium (-FCS) and poured dropwise onto cells. After 8 hours, the medium was changed to DME medium (+FCS), and cells were recovered 36 hours later. A portion of the cells was mixed with a sample buffer and subjected to SDS-PAGE. The expression of each gene from the co-transfected construct was then confirmed by immunodetection using Western blot transfer, anti-MYC monoclonal antibody, and anti-HA monoclonal antibody. The remaining cells were vortexed, suspended in TNE buffer [10 mM Tris-HCl (pH 8.5), 1 mM EDTA, 0.2% NP-40, 1 mM DTT, 2 mM benzamidine-HCl, 250 mM NaCl, 10 µg/ml aprotin and 100 mM PMSF) using a rotary mixer, and centrifuged to remove sedimented debris.

Pre-clearing was performed to remove proteins binding to protein A Sepharose and IgG contained in the recovered cell suspension. Protein A Sepharose and normal rabbit IgG were mixed, centrifuged and washed to recover protein A Sepharose-IgG. Protein A Sepharose-IgG and cell suspension were mixed, and proteins non-specifically binding to protein A Sepharose-IgG were sedimented by centrifugation. The supernatant thus obtained was used as the sample in immunoprecipitation experiments.

The pre-cleared cell suspension was divided into two half portions. Anti-MYC monoclonal antibody and normal rabbit IgG were added to each portion, and they were mixed by O/N, and further mixed with protein A Sepharose. An "Anti-MYC monoclonal antibody"-"6myc-epitope-tagg d-TRAF4"-"HA-epitope-tagged-TFAF1/2-TRAF4" conjugat or normal rabbit IgG bound to protein A Sepharose was sedimented by

centrifugation and washed with NETN buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5% NP-40 and 150 mM NaCl). This sediment was dissolved in a sample buffer, electrophoresed, and transferred to a membrane HA-epitope-tagged TFAF1, HA-epitope-tagged TFAF2, and TRAF4 were then detected by immunodetection using anti-HA monoclonal antibody. The results shown in Fig. 6 demonstrate that, under the forced expression in *in vivo* system, TRAF4 associates with TFAF1 or TFAF2, and that TRAF4 forms a homodimer.

7.3 Association between TRAFs and TFAF1 (*in vivo* immunoprecipitation)

Although TFAF1 was cloned as a factor binding to TRAF4, the binding of TFAF1 to TRAF4 may not be specific because TFAF1, 2, 3, 4, 5 and 6 have homologous protein structures. Therefore, the 293T cell line derived from human fetal renal cell was co-transfected with CMV6myc-TRAFs (six genes, 1, 2, 3, 4, 5 and 6) consturcted by re-cloning TRAFs into an myc-epitope-tag vector pCMV6myc and pcDNAha-TFAF1 constructed by re-cloning TFAF1 into an HA-epitope-tag vector pcDNAha. Their intracellular association when each of them was forcibly expressed was confirmed by *in vivo* immunoprecipitation experiments. Anti-TFAF1 polyclonal antibody was used for immunoprecipitation, and anti-MYC monoclonal antibody was employed for immunodetection. Experiments were then performed by similar procedures as described in the section [Association between TRAF4 and TFAFs (*in vivo* immunoprecipitation)]. The results shown in Fig. 7 confirm that, under the forcible expression, TRAF4 associates with TFAF1 more specifically than with other TRAFs. Besides TRAF4, a weak

association of TRAF2 with TFAF1 was observed.

8. Examination of Amino Acid Sequence Homology of TFAF1 and TFAF2 (Fig. 8)

In order to assume functions of TFAF1/2 from their amino acid seqences, amino acid sequence homology was examined using the www service (http://www.genome.ad.jp) of the Human Genome Analysis Center of the Institute of Medical Science, University of Tokyo and the Super-computer Laboratory of the Institute of Chemical Research, Kyoto University.

8.1 Sequence homology search program BLAST

Using the non-redundant amino acid sequence data base nr-aa, amino acid sequences homologous to those of TFAF1/2 were searched (blastp search). No sequence homologous to that of TFAF1 was found. TFAF2 had homology with Sorting Nexin 1 (SNX1), a factor involved in the intracytoplasmic transportation of proteins, over a wide range, even though weak. The sequence alignment of both is shown in Fig. 8. No specific motif was found in TFAF1. It was confirmed that TFAF2 had a leucine zipper structure at the N-terminal region.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.

(ii) TITLE OF INVENTION: TRAF4-ASSOCIATING PROTEIN

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: ASCII (text)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: CA 2,245,340

(B) FILING DATE: 19-AUG-1998

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: SMART & BIGGAR

(B) REGISTRATION NUMBER:

(C) REFERENCE/DOCKET NUMBER: 76432-17

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (613)-232-2486

(B) TELEFAX: (613)-232-8440

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1530 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESSS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: human placenta cDNA library

(B) CLONE: #127

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 106..822

(C) IDENTIFICATION METHOD: by experiment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

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GCCGGTGGCA CGACAGTTGC TGCAGGGAAT CTTTTAAACG AGAGCGAGAA GGACTGCGGG     60
CAGGACCGGC GGGCTCCTGG GGTTCAGCCG TGCCGCCTCG TTACG ATG ACC AGT GTG    117
                                                    Met Thr Ser Val
                                                      1

GTT AAG ACA GTG TAT AGC CTG CAG CCC CCC TCT GCG CTG AGC GGC GGC      165
Val Lys Thr Val Tyr Ser Leu Gln Pro Pro Ser Ala Leu Ser Gly Gly
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5                   10                  15                  20

CAG CCG GCA GAC ACA CAA ACT CGG GCC ACT TCT AAG AGT CTC TTA CCT    213
Gln Pro Ala Asp Thr Gln Thr Arg Ala Thr Ser Lys Ser Leu Leu Pro
                25                  30                  35

GTT AGG TCC AAA GAA GTC GAT GTT TCC AAA CAG CTT CAT TCA GGA GGT    261
Val Arg Ser Lys Glu Val Asp Val Ser Lys Gln Leu His Ser Gly Gly
            40                  45                  50

CCA GAG AAT GAT GTT ACA AAA ATC ACC AAA CTG AGA CGA GAG AAT GGG    309
Pro Glu Asn Asp Val Thr Lys Ile Thr Lys Leu Arg Arg Glu Asn Gly
        55                  60                  65

CAA ATG AAA GCT ACT GAC ACT GCC ACC AGA AGG AAT GTC AGA AAA GGC    357
Gln Met Lys Ala Thr Asp Thr Ala Thr Arg Arg Asn Val Arg Lys Gly
    70                  75                  80

TAC AAA CCA CTG AGT AAG CAA AAA TCA GAG GAA GAG CTC AAG GAC AAG    405
Tyr Lys Pro Leu Ser Lys Gln Lys Ser Glu Glu Glu Leu Lys Asp Lys
85                  90                  95                  100

AAC CAG CTG TTA GAA GCC GTC AAC AAG CAG TTG CAC CAG AAG TTG ACT    453
Asn Gln Leu Leu Glu Ala Val Asn Lys Gln Leu His Gln Lys Leu Thr
                105                 110                 115

GAA ACT CAG GGA GAG CTG AAG GAC CTG ACC CAG AAG GTA GAG CTG CTG    501
Glu Thr Gln Gly Glu Leu Lys Asp Leu Thr Gln Lys Val Glu Leu Leu
            120                 125                 130

GAG AAG TTT CGG GAC AAC TGT TTG GCA ATT TTG GAG AGC AAG GGC CTT    549
Glu Lys Phe Arg Asp Asn Cys Leu Ala Ile Leu Glu Ser Lys Gly Leu
        135                 140                 145

GAT CCA GCT TTA GGC AGT GAG ACC CTG TCA TCA CGA CAA GAA TCC ACT    597
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Asp Pro Ala Leu Gly Ser Glu Thr Leu Ser Ser Arg Gln Glu Ser Thr
    150                 155                 160
ACT GAT CAC ATG GAC TCT ATG TTG CTG TTA GAA ACT TTG CAA GAG GAG    645
Thr Asp His Met Asp Ser Met Leu Leu Leu Glu Thr Leu Gln Glu Glu
165                 170                 175                 180
CTG AAG CTT TTT AAC GAA ACA GCC AAA AAG CAG ATG GAG GAG TTA CAG    693

Leu Lys Leu Phe Asn Glu Thr Ala Lys Lys Gln Met Glu Glu Leu Gln
                185                 190                 195
GCC TTA AAG GTA AAG CTG GAG ATG AAA GAG GAA AGA GTC CGA TTC CTA    741
Ala Leu Lys Val Lys Leu Glu Met Lys Glu Glu Arg Val Arg Phe Leu
            200                 205                 210
GAA CAG CAA ACC TTA TGT AAC AAT CAA GTA AAT GAT TTA ACA ACA GCC    789
Glu Gln Gln Thr Leu Cys Asn Asn Gln Val Asn Asp Leu Thr Thr Ala
        215                 220                 225
CTT AAG GAA ATG GAG CAG CTA TTA GAA ATG TAA GAAGAAGCAA GTGGCCAGAT   842
Leu Lys Glu Met Glu Gln Leu Leu Glu Met
    230                 235
GGCTCCCTCT TGGGCATAAA ATCTCAGAGG AGGCTACTTA GGACATCATC TTGGCCATGA  902
TCTTCTGGGA CTCACCATCT CCAGAATGAA AACAATTTCT ACAGTAGACT TAAGGACAGT  962
TTATGCTGAA ATGGCAATTC CTCATTTAAG CAAGTTTTCC CAACCTTCAG GTTGGTCAGC 1022
CCTCCTGAGC CTCACAGGTG GATAATTGAG GCCTACAAGA GAGGGGAGCC TAGGAGCTTG 1082
GATTGACTTT CTAGTCAACC ACCTGACTCC AGCACACCAT TACAATCGGG AGACTAAACC 1142
AACAACCAGA GGATCTAAAA TGTCACATTC AGATTTTCAG GAAGAAAATC TTCATTACAG 1202
TGGAGCACAA ATGTTCCATA CAAGACATCA TTGAGGAGCC ATGCTGTCCC TTTCTAACCT 1262
GAAACACATT CTTTCCCATC CTGGTTGGGC TTCTGTACCT CCTTATTAAT TTATGAACCT 1322
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GAAGTTGCTT GAAGTGTTTT GGGCTTAATA AATGGGGTGA AAGTATAGGT AGCAGTAACA 1382

CCTACATGAA ACAATACACC TTGGATCTTT TAATCTAAAT TACTTTTCTT TTTTAAGTCT 1442

ACTTTTAAAA TAAATACTTC TGTAAATATT CTGACTGTAA CATTGAGGAA TGAAAATAGC 1502

CTTTTAACCT AAAAAAAAAA AAAAAAAA 1530

(2)INFORMATION FOR SEQ ID NO: 2:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 238 amino acids

(B)TYPE: amino acid

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: protein

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 2

Met Thr Ser Val Val Lys Thr Val Tyr Ser Leu Gln Pro Pro Ser Ala

1 5 10 15

Leu Ser Gly Gly Gln Pro Ala Asp Thr Gln Thr Arg Ala Thr Ser Lys

20 25 30

Ser Leu Leu Pro Val Arg Ser Lys Glu Val Asp Val Ser Lys Gln Leu

35 40 45

His Ser Gly Gly Pro Glu Asn Asp Val Thr Lys Ile Thr Lys Leu Arg

50 55 60

Arg Glu Asn Gly Gln Met Lys Ala Thr Asp Thr Ala Thr Arg Arg Asn

65 70 75 80

Val Arg Lys Gly Tyr Lys Pro Leu Ser Lys Gln Lys Ser Glu Glu Glu

85 90 95

Leu Lys Asp Lys Asn Gln Leu Leu Glu Ala Val Asn Lys Gln Leu His

100 105 110

Gln Lys Leu Thr Glu Thr Gln Gly Glu Leu Lys Asp Leu Thr Gln Lys
115 120 125
Val Glu Leu Leu Glu Lys Phe Arg Asp Asn Cys Leu Ala Ile Leu Glu
130 135 140
Ser Lys Gly Leu Asp Pro Ala Leu Gly Ser Glu Thr Leu Ser Ser Arg
145 150 155 160
Gln Glu Ser Thr Thr Asp His Met Asp Ser Met Leu Leu Leu Glu Thr
165 170 175
Leu Gln Glu Glu Leu Lys Leu Phe Asn Glu Thr Ala Lys Lys Gln Met
180 185 190
Glu Glu Leu Gln Ala Leu Lys Val Lys Leu Glu Met Lys Glu Glu Arg
195 200 205
Val Arg Phe Leu Glu Gln Gln Thr Leu Cys Asn Asn Gln Val Asn Asp
210 215 220
Leu Thr Thr Ala Leu Lys Glu Met Glu Gln Leu Leu Glu Met
225 230 235

(2)INFORMATION FOR SEQ ID NO: 3:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 1906 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESSS: double

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: cDNA to mRNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: Homo sapiens

(vii)IMMEDIATE SOURCE:

(A)LIBRARY: human placenta cDNA library

(B)CLONE: #293

(ix)FEATURE:

(A)NAME/KEY: CDS

(B)LOCATION: 13..1233

(C)IDENTIFICATION METHOD: by experiment

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 3

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CTCGGAGCAG CC ATG ATG GAA GGC CTG GAC GAC GGC CCG GAC TTC CTC TCA  51
              Met Met Glu Gly Leu Asp Asp Gly Pro Asp Phe Leu Ser
                1               5                  10

GAA GAG GAC CGC GGA CTT AAA GCA ATA AAT GTA GAT CTT CAA AGT GAT    99
Glu Glu Asp Arg Gly Leu Lys Ala Ile Asn Val Asp Leu Gln Ser Asp
     15                  20                  25

GCT GCT CTG CAG GTG GAC ATT TCT GAT GCT CTT AGT GAG CGG GAT AAA   147
Ala Ala Leu Gln Val Asp Ile Ser Asp Ala Leu Ser Glu Arg Asp Lys
 30                  35                  40                  45

GTA AAA TTC ACT GTT CAC ACA AAG AGT TCA TTG CCA AAT TTT AAA CAA   195
Val Lys Phe Thr Val His Thr Lys Ser Ser Leu Pro Asn Phe Lys Gln
                 50                  55                  60

AAC GAG TTT TCA GTT GTT CGG CAA CAT GAG GAA TTT ATC TGG CTT CAT   243
Asn Glu Phe Ser Val Val Arg Gln His Glu Glu Phe Ile Trp Leu His
            65                  70                  75

GAT TCC TTT GTT GAA AAT GAA GAC TAT GCA GGT TAT ATC ATT CCA CCA   291
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Asp Ser Phe Val Glu Asn Glu Asp Tyr Ala Gly Tyr Ile Ile Pro Pro
         80                  85                  90

GCA CCA CCA AGA CCT GAT TTT GAT GCT TCA AGG GAA AAA CTA CAG AAG   339
Ala Pro Pro Arg Pro Asp Phe Asp Ala Ser Arg Glu Lys Leu Gln Lys
     95                 100                 105

CTT GGT GAA GGA GAA GGG TCA ATG ACG AAG GAA GAA TTC ACA AAG ATG   387
Leu Gly Glu Gly Glu Gly Ser Met Thr Lys Glu Glu Phe Thr Lys Met
110                 115                 120                 125

AAA CAG GAA CTG GAA GCT GAA TAT TTG GCA ATA TTC AAG AAG ACA GTT   435
Lys Gln Glu Leu Glu Ala Glu Tyr Leu Ala Ile Phe Lys Lys Thr Val
                130                 135                 140

GCG ATG CAT GAA GTG TTC CTG TGT CGT GTG GCA GCA CAT CCT ATT TTG   483
Ala Met His Glu Val Phe Leu Cys Arg Val Ala Ala His Pro Ile Leu
            145                 150                 155

AGA AGA GAT TTA AAT TTC CAT GTC TTC TTG GAA TAT AAT CAA GAT TTG   531
Arg Arg Asp Leu Asn Phe His Val Phe Leu Glu Tyr Asn Gln Asp Leu
        160                 165                 170

AGT GTG CGA GGA AAA AAT AAA AAA GAG AAA CTT GAA GAC TTC TTT AAA   579
Ser Val Arg Gly Lys Asn Lys Lys Glu Lys Leu Glu Asp Phe Phe Lys
    175                 180                 185

AAC ATG GTT AAA TCA GCA GAT GGA GTA ATC GTT TCA GGA GTA AAG GAT   627
Asn Met Val Lys Ser Ala Asp Gly Val Ile Val Ser Gly Val Lys Asp
190                 195                 200                 205

GTA GAT GAT TTC TTT GAG CAC GAA CGA ACA TTT CTT TTG GAA TAT CAT   675
Val Asp Asp Phe Phe Glu His Glu Arg Thr Phe Leu Leu Glu Tyr His
                210                 215                 220
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AAC CGA GTT AAG GAT GCA TCT GCT AAA TCT GAT AGA ATG ACA AGA TCC    723
Asn Arg Val Lys Asp Ala Ser Ala Lys Ser Asp Arg Met Thr Arg Ser
            225                 230                 235

CAC AAA AGT GCT GCA GAT GAT TAC AAT AGA ATT GGT TCT TCA TTA TAT    771
His Lys Ser Ala Ala Asp Asp Tyr Asn Arg Ile Gly Ser Ser Leu Tyr
        240                 245                 250

GCT TTA GGA ACT CAG GAT TCT ACA GAT ATA TGC AAG TTT TTT CTC AAA    819
Ala Leu Gly Thr Gln Asp Ser Thr Asp Ile Cys Lys Phe Phe Leu Lys
    255                 260                 265

GTT TCA GAA CTG TTC GAT AAA ACA AGA AAA ATA GAA GCA CGA GTG TCT    867
Val Ser Glu Leu Phe Asp Lys Thr Arg Lys Ile Glu Ala Arg Val Ser
270                 275                 280                 285

GCT GAT GAA GAC CTC AAA CTT TCT GAT CTT TTA AAA TAT TAC TTA AGA    915
Ala Asp Glu Asp Leu Lys Leu Ser Asp Leu Leu Lys Tyr Tyr Leu Arg
                290                 295                 300

GAA TCT CAA GCT GCT AAG GAT CTC CTG TAT CGA AGG TCT AGG TCA CTA    963
Glu Ser Gln Ala Ala Lys Asp Leu Leu Tyr Arg Arg Ser Arg Ser Leu
            305                 310                 315

GTG GAT TAT GAA AAT GCT AAT AAA GCA CTG GAT AAA GCA AGA GCA AAA   1011
Val Asp Tyr Glu Asn Ala Asn Lys Ala Leu Asp Lys Ala Arg Ala Lys
        320                 325                 330

AAT AAA GAT GTT CTA CAG GCC GAA ACT TCC CAA CAA TTA TGT TGT CAG   1059
Asn Lys Asp Val Leu Gln Ala Glu Thr Ser Gln Gln Leu Cys Cys Gln
    335                 340                 345

AAA TTT GAA AAA ATA TCT GAG TCT GCA AAA CAA GAA CTT ATA GAT TTT   1107
Lys Phe Glu Lys Ile Ser Glu Ser Ala Lys Gln Glu Leu Ile Asp Phe
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350                 355                 360                 365

AAG ACA AGA AGA GTT GCT GCA TTC AGA AAA AAT TTA GTG GAA CTG GCA   1155
Lys Thr Arg Arg Val Ala Ala Phe Arg Lys Asn Leu Val Glu Leu Ala

                370                 375                 380

GAG TTA GAA CTG AAG CAT GCA AAG GGT AAT CTA CAG TTG CTG CAG AAC   1203
Glu Leu Glu Leu Lys His Ala Lys Gly Asn Leu Gln Leu Leu Gln Asn

            385                 390                 395

TGC CTG GCA GTG TTA AAT GGA GAC ACA TAA GCCACACTCC GCCTTCCTGT     1253
Cys Leu Ala Val Leu Asn Gly Asp Thr

        400                 405

TAAAAAGGGC TGCCTTCCTT CAAATTTTAT TTTTGTTTTC TTAATGATGT TAAGCATTTA 1313

TGCTCACTGG AAACAAACAA AAAGCAGCTG AAAAAGTGCA TCAACTCCTC TTTTTCTGAG 1373

AAACATGGAG CAGCGCACGC CCAGGCGATG CCAGTCTGTG TGCCGTGATG CCGCACTGTG 1433

TTCCCCATGA CAGTGATCCA TCATCGTGCA CTCGTCATAC TCAGAAGTCC AAAGTTCATT 1493

CTTCTTTAAA GTAGCCTCTA TAACTCTGTT TATTTTATAA ATAGTATTCC TTATGGCTGC 1553

CACTCTTATT TACCTTTAAA TAATTTCTGA AATTTAACCT TTTCAGAATG CATTGTTGAA 1613

ACAAGATAAA GATTGCCTTT TTTGAATTTT TTAAATTTTG TTTTTAAAAG CATATACCAC 1673

CTTAGTTCAT TCATGTATCC TGGTAAAGCA TCTTAATCAG ACTTATTTTT AATTACTGGA 1733

TATTTTTTAG ACGTTTTGGG ACAGATTTTA TGTAATTTTT ATAAGTATGA TTTTTGGAGG 1793

AAAGCAAATG CATTAGTATG TTTGCCTTAA AATTGTAGAC TAAACCAAGT ATTGTAAAAT 1853

AAACAGCGAT AACCGTGGAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAA         1906
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(2)INFORMATION FOR SEQ ID NO: 4:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 406 amino acids

(B)TYPE: amino acid

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: protein

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 4

Met Met Glu Gly Leu Asp Asp Gly Pro Asp Phe Leu Ser Glu Glu Asp
1 5 10 15

Arg Gly Leu Lys Ala Ile Asn Val Asp Leu Gln Ser Asp Ala Ala Leu
20 25 30

Gln Val Asp Ile Ser Asp Ala Leu Ser Glu Arg Asp Lys Val Lys Phe
35 40 45

Thr Val His Thr Lys Ser Ser Leu Pro Asn Phe Lys Gln Asn Glu Phe
50 55 60

Ser Val Val Arg Gln His Glu Glu Phe Ile Trp Leu His Asp Ser Phe
65 70 75 80

Val Glu Asn Glu Asp Tyr Ala Gly Tyr Ile Ile Pro Pro Ala Pro Pro
85 90 95

Arg Pro Asp Phe Asp Ala Ser Arg Glu Lys Leu Gln Lys Leu Gly Glu
100 105 110

Gly Glu Gly Ser Met Thr Lys Glu Glu Phe Thr Lys Met Lys Gln Glu
115 120 125

Leu Glu Ala Glu Tyr Leu Ala Ile Phe Lys Lys Thr Val Ala Met His
130 135 140

Glu Val Phe Leu Cys Arg Val Ala Ala His Pro Ile Leu Arg Arg Asp
145 150 155 160

Leu Asn Phe His Val Phe Leu Glu Tyr Asn Gln Asp Leu Ser Val Arg
165 170 175

Gly Lys Asn Lys Lys Glu Lys Leu Glu Asp Phe Phe Lys Asn Met Val

180 185 190

Lys Ser Ala Asp Gly Val Ile Val Ser Gly Val Lys Asp Val Asp Asp

195 200 205

Phe Phe Glu His Glu Arg Thr Phe Leu Leu Glu Tyr His Asn Arg Val

210 215 220

Lys Asp Ala Ser Ala Lys Ser Asp Arg Met Thr Arg Ser His Lys Ser

225 230 235 240

Ala Ala Asp Asp Tyr Asn Arg Ile Gly Ser Ser Leu Tyr Ala Leu Gly

245 250 255

Thr Gln Asp Ser Thr Asp Ile Cys Lys Phe Phe Leu Lys Val Ser Glu

260 265 270

Leu Phe Asp Lys Thr Arg Lys Ile Glu Ala Arg Val Ser Ala Asp Glu

275 280 285

Asp Leu Lys Leu Ser Asp Leu Leu Lys Tyr Tyr Leu Arg Glu Ser Gln

290 295 300

Ala Ala Lys Asp Leu Leu Tyr Arg Arg Ser Arg Ser Leu Val Asp Tyr

305 310 315 320

Glu Asn Ala Asn Lys Ala Leu Asp Lys Ala Arg Ala Lys Asn Lys Asp

325 330 335

Val Leu Gln Ala Glu Thr Ser Gln Gln Leu Cys Cys Gln Lys Phe Glu

340 345 350

Lys Ile Ser Glu Ser Ala Lys Gln Glu Leu Ile Asp Phe Lys Thr Arg

355 360 365

Arg Val Ala Ala Phe Arg Lys Asn Leu Val Glu Leu Ala Glu Leu Glu

370 375 380

Leu Lys His Ala Lys Gly Asn Leu Gln Leu Leu Gln Asn Cys Leu Ala

385 390 395 400

Val Leu Asn Gly Asp Thr

405

(2)INFORMATION FOR SEQ ID NO: 5:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 26

(B)TYPE: nucleic acid

(C)STRANDEDNESSS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid

(A)DESCRIPTION: synthetic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 5

ACGGATCCTG CCCTAAGCTG GCAATG 26

(2)INFORMATION FOR SEQ ID NO: 6:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 26

(B)TYPE: nucleic acid

(C)STRANDEDNESSS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid

(A)DESCRIPTION: synthetic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 6

TGCTCGAGCC TGCACTCAGC TGAGGA 26

What Is Claimed Is:

1. A protein comprising the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4, or a protein comprising an amino acid sequence having one or more amino acids substituted, deleted or added in the amino acid sequence of said protein, and having the activity to associate with TNF recepter associated factor 4.

2. A protein encoded by a DNA hybridizing with a DNA comprising the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3, wherein said protein has the activity to associate with TNF recepter associated factor 4.

3. A DNA encoding the protein of claim 1.

4. The DNA of claim 3 which comprises the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3.

5. A DNA hybridizing with a DNA comprising the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3, wherein said DNA encodes a protein having the activity to associate with TNF recepter associated factor 4.

6. A vector comprising the DNA of claim 3.

7. A transformant harboring the vector of claim 6.

8. A method for preparing the protein of claim 1, comprisng a step of culturing the transformant of claim 7.

9. An antisense DNA corresponding to the DNA of claim 4 or a portion thereof.

10. An antibody binding to the protein of claim 1 or 2.

11. A method for screening a compound having the activity to

suppress the association of TNF receptor associated factor 4 with an associated factor thereof, comprisng

(a) contacting a candidate substance and TNF receptor associated factor 4 with the protein of claim 1 or 2, and

(b) quantitating the protein of claim 1 which has associated and/or not associated with TNF receptor associated factor 4.

12. The method of claim 11, wherein in step (a) the candidate substance and TNF receptor associated factor 4 are simultaneously contacted with the protein according to claim 1 or 2.

13. The method of claim 11, wherein in step (a) the candidate substance is first contacted with TNF receptor associated factor 4 and then with the protein of claim 1 or 2.

Smart & Biggar
Ottawa, Canada
Patent Agents

Fig. 1

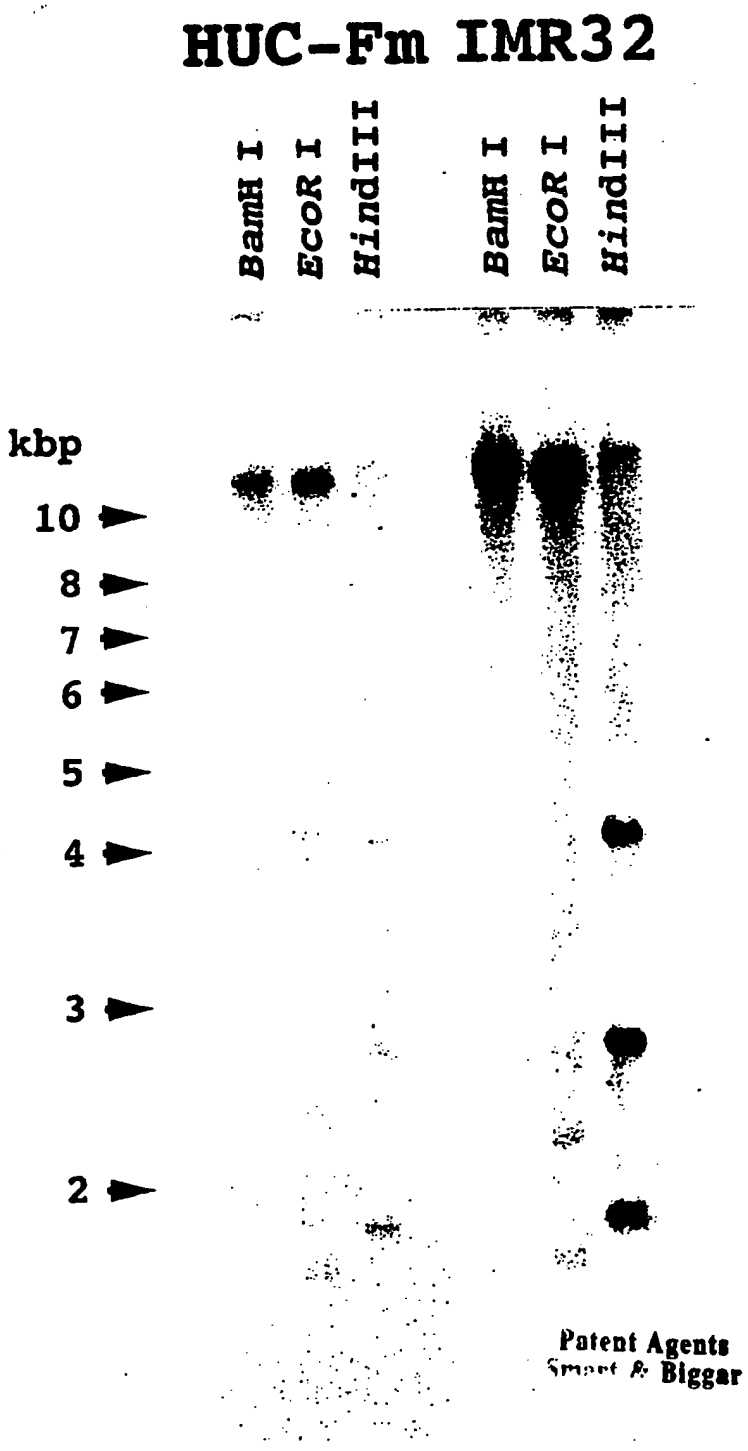


Fig. 2

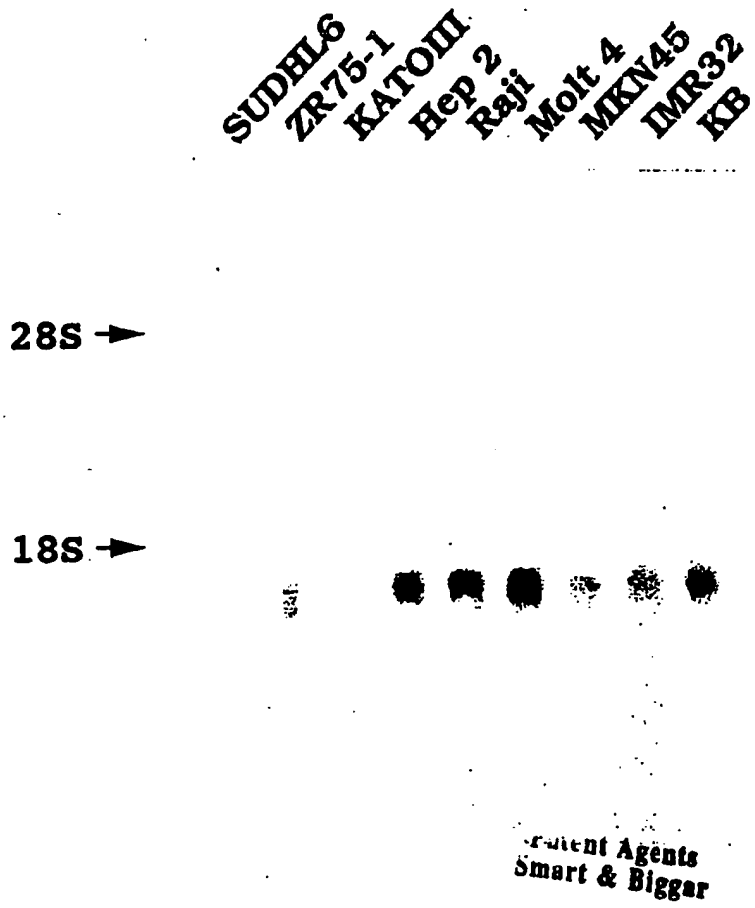
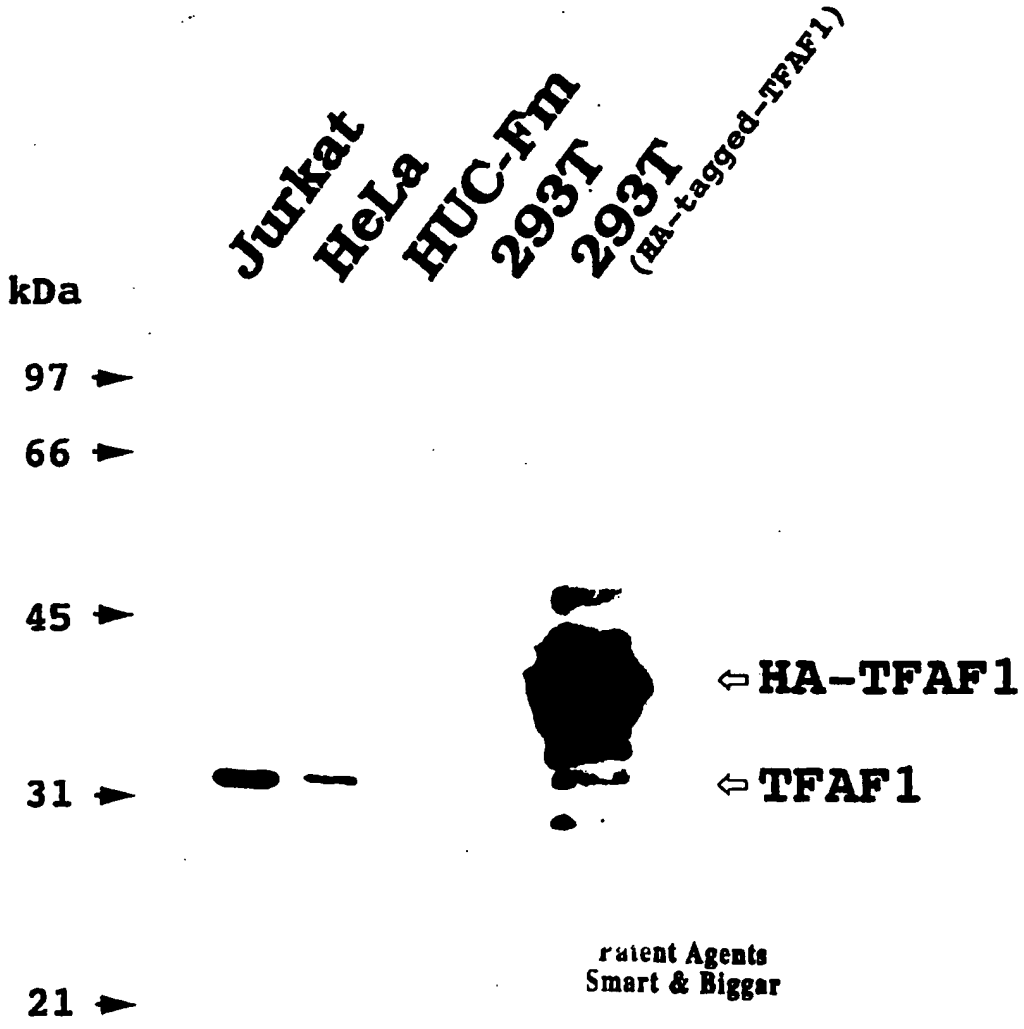


Fig. 3



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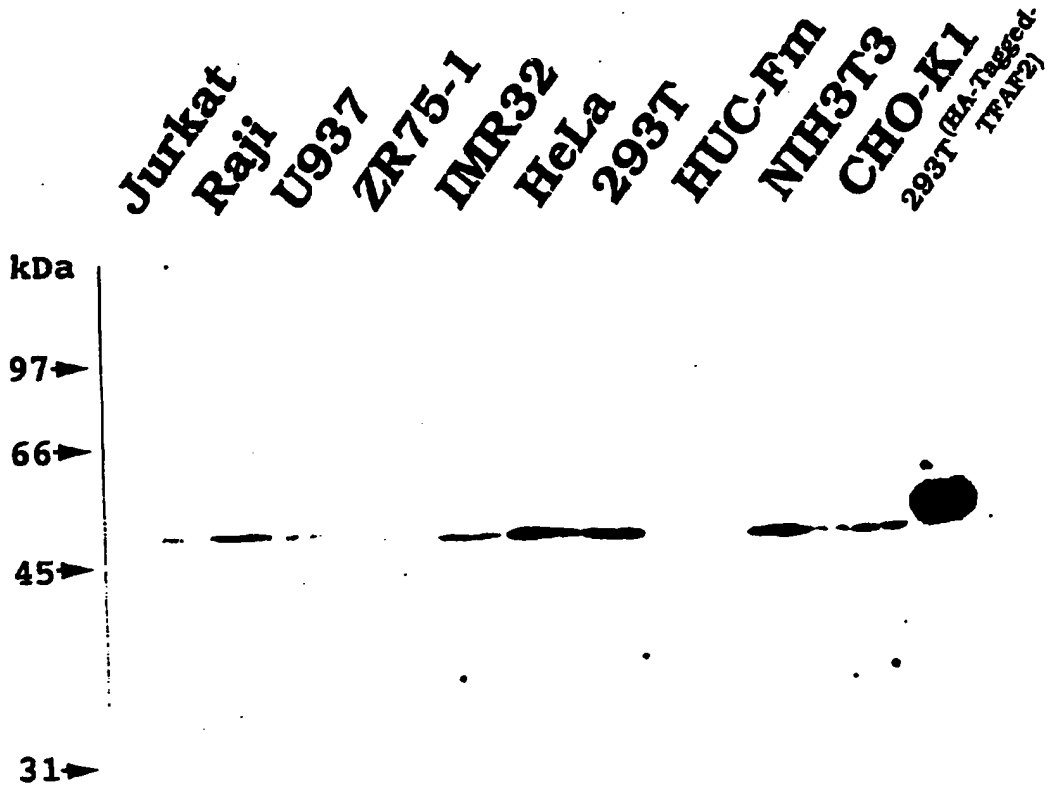
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Raji
U937
ZR75-1
IMR32
HeLa
293T
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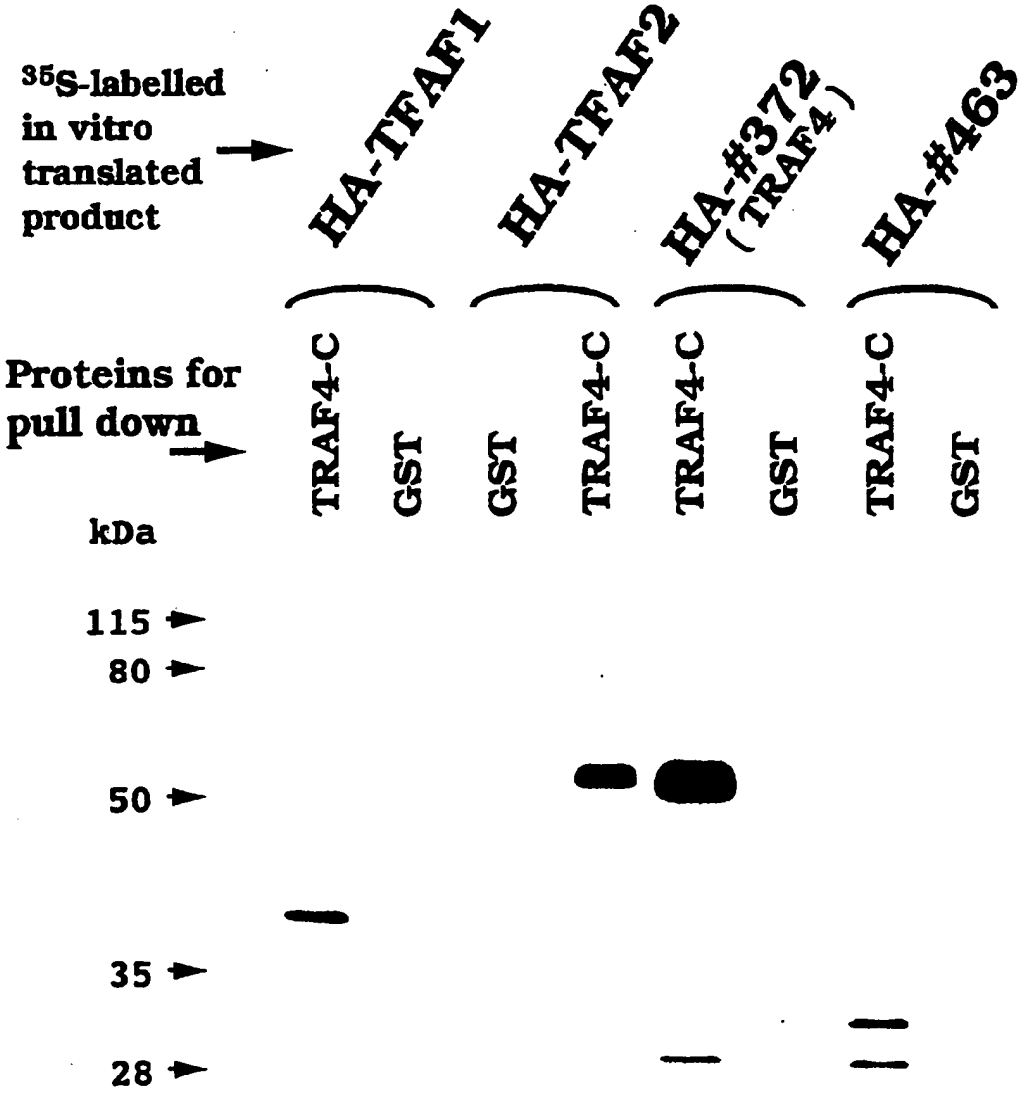
NIH3T3
CHO-K1
293T (HA-Tagged TFAP2)
kDa
97
66
45
31

Fig. 5


35S-labelled in vitro translated product
HA-TFAF1
HA-TFAF2
HA-#372 (TRAF4)
HA-#463
Proteins for pull down
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GST
GST
TRAF4-C
TRAF4-C
GST
TRAF4-C
GST
kDa
115
80
50
35
28

Fig. 6

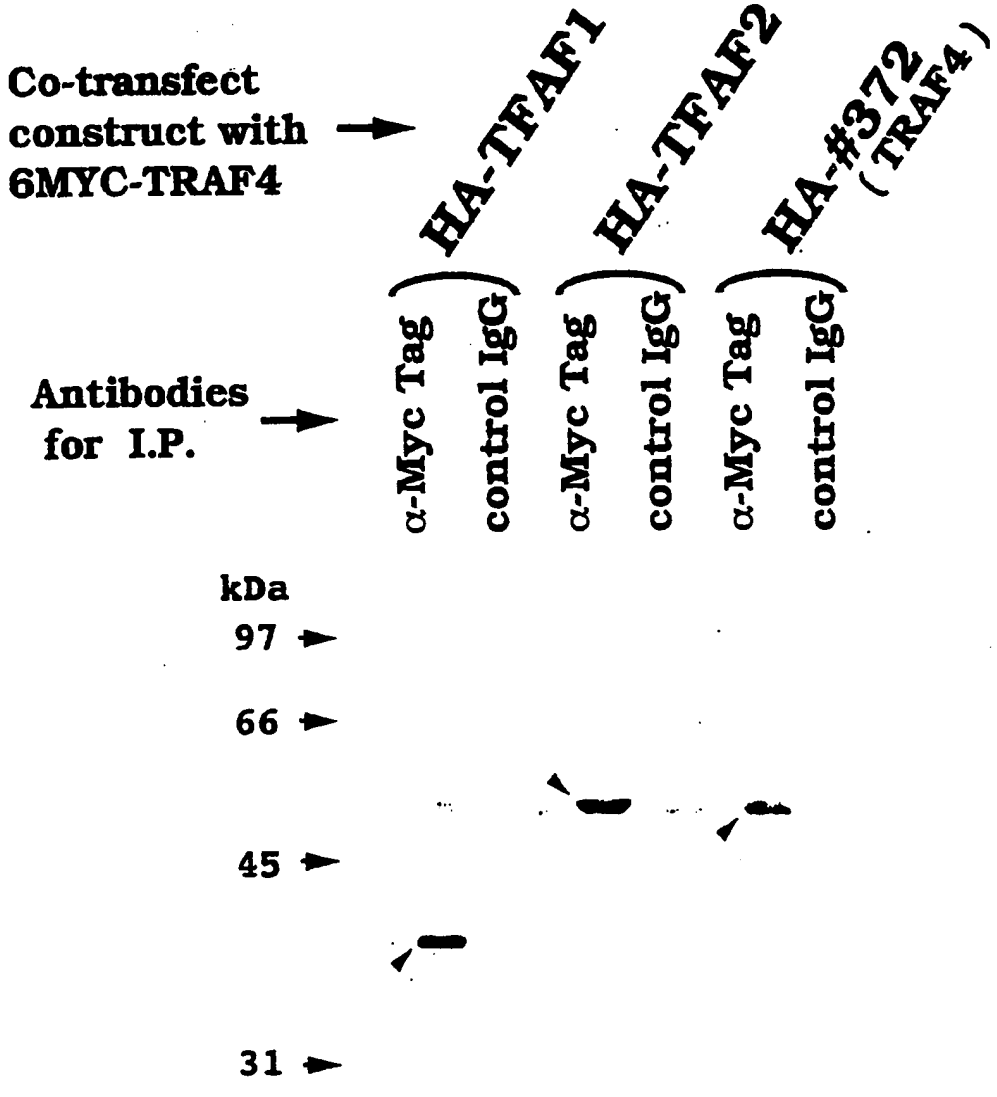

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Fig. 7

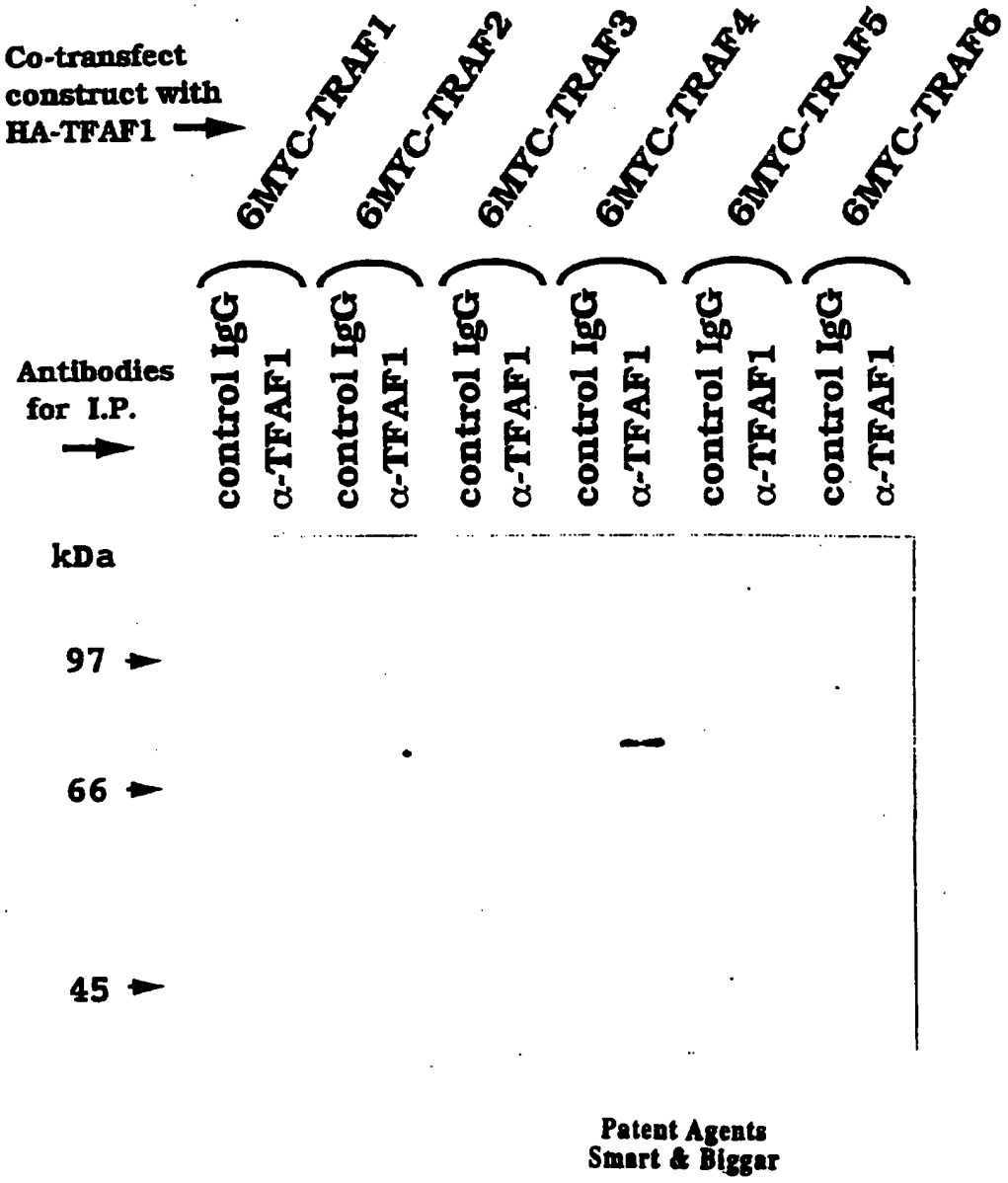
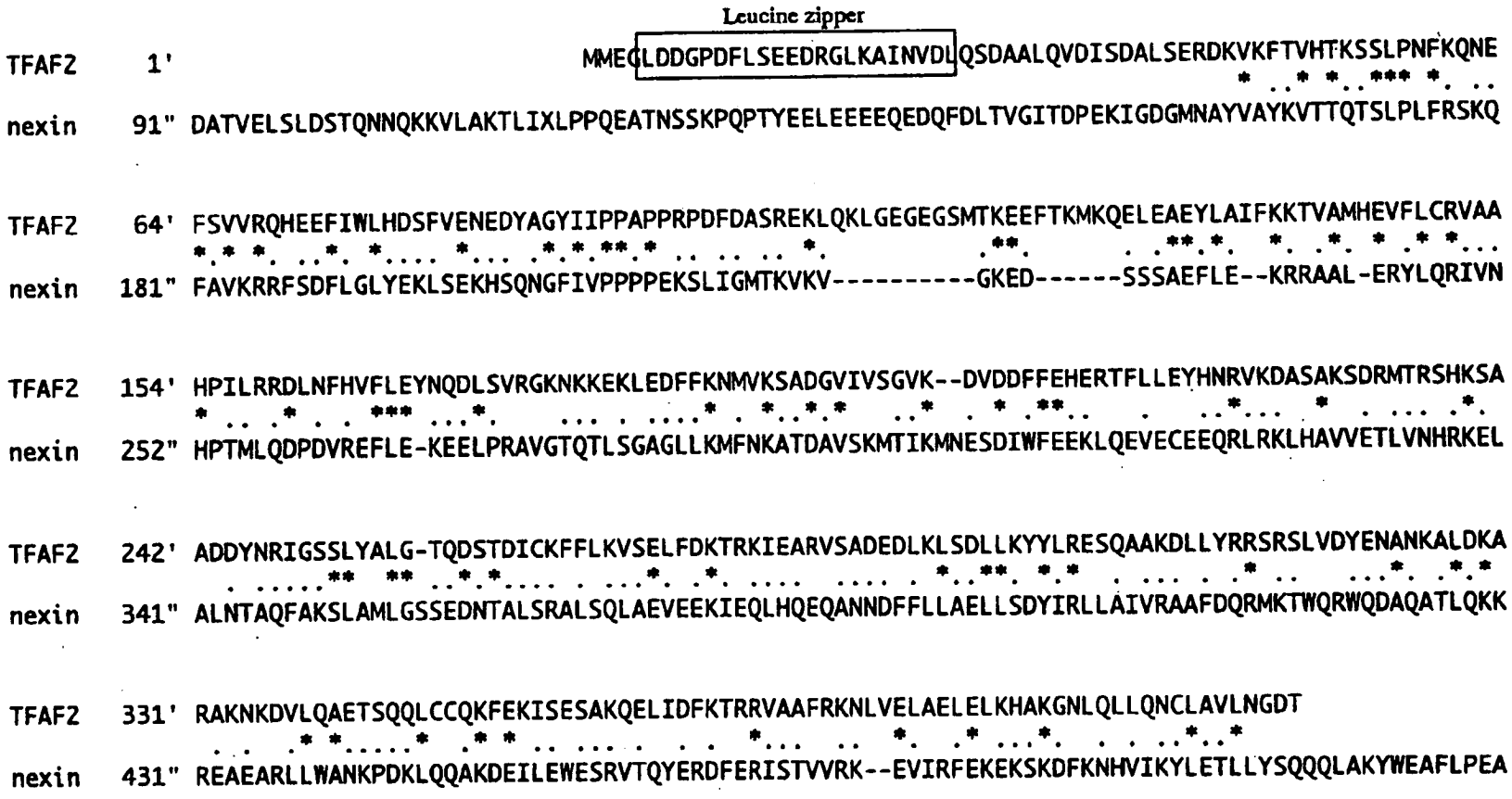
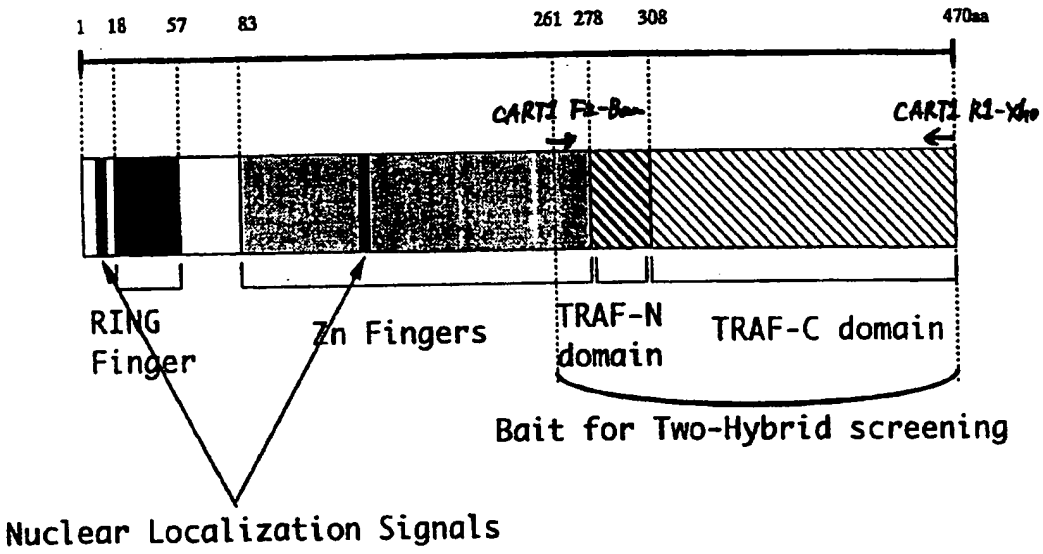


Fig.8



CA 02245340 1998-08-19

Fig. 9



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